

WO04083373

Publication Title:

WO04083373

Abstract:

Abstract not available for WO04083373

Data supplied from the esp@cenet database - Worldwide

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(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
30 September 2004 (30.09.2004)

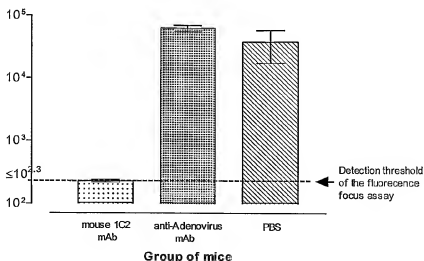
PCT

(10) International Publication Number
WO 2004/083373 A2

- (51) International Patent Classification⁷: C12N
- (21) International Application Number: PCT/GB2004/001239
- (22) International Filing Date: 22 March 2004 (22.03.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 0306618.0 22 March 2003 (22.03.2003) GB
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GI, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Declaration under Rule 4.17:**
— of inventorship (Rule 4.17(iv)) for US only
- Published:**
— without international search report and to be republished upon receipt of that report

[Continued on next page]

(54) Title: ANTIBODY



(57) Abstract: The present invention relates to an antibody, or a functional derivative thereof, against the G-glycoprotein of Respiratory Syncytial Virus (RSV) with a variable region comprising a first domain from a V_L region and a second domain from a V_H region. The invention also relates to DNA molecules encoding such an antibody and also cells transformed with such DNA molecules.

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ANTIBODY

The present invention relates to antibodies and in particular to antibodies that may be used to treat or prevent the development of infections caused by the Respiratory Syncytial Virus (RSV).

Human RSV is a member of the *Pneumovirus* genus, *Pneumovirinae* subfamily in the *Paramyxoviridae* family. Other members of the genus are bovine RSV (BRSV), ovine RSV (ORSV), caprine RSV (CRSV), and pneumonia virus of mice (PVM).

RSV particles are pleiomorphic and enveloped. The particles are usually spherical (approximately 80-500 nm in diameter) but filamentous forms with a diameter of 60-100 nm and up to 10 μ m in length are also seen budding from the surface of infected cells. The virions consist of a helical nucleocapsid 11-15 nm in diameter, surrounded by an envelope from which 12 nm-long glycoprotein spikes protrude. The viral envelope is composed of a host plasma membrane-derived lipid bilayer that contains virally encoded transmembrane proteins. Replication of RSV takes place in the cytoplasm, and RSV virions are assembled at the cytoplasm membrane.

Different isolates or laboratory strains of human RSV are divided into two groups, A and B. This division is based mainly on their differing reactivities to panels of monoclonal antibodies, particularly those directed against the phosphoprotein (P), fusion protein (F) and attachment (G) glycoprotein of RSV.

The RSV genome is a 5×10^6 Dalton, non-segmented single-stranded negative sense RNA molecule contained within the nucleocapsid of the virion. The genomic RNA is neither capped nor polyadenylated. Although pneumoviruses encode a larger number of mRNAs than the other paramyxoviruses, the length of the genomic RNA is not much different. The RSV genome is transcribed into 10 mRNA molecules during virus replication, each of which codes for a single protein, except the M2 mRNA which encodes two separate proteins. The genome order of RSV (represented by the strain A2, 15, 222 nucleotides) is as follows: 3' NS1 - NS2 - N - P - M - SH - G - F - M2-1 (22K), 2 (11K) - L 5'.

Eight or nine of the polypeptides are structural proteins found within the RSV virion. The nucleocapsid protein (N), phosphoprotein (P), transcription anti-termination factor (M2-1), and large protein (L, the virion RNA dependent RNA polymerase), together with the RNA genome, make up the virus nucleocapsid. The fusion glycoprotein (F), attachment glycoprotein (G), and small hydrophobic protein (SH) are transmembrane proteins located in the virion envelope; F and G form the glycoprotein spikes seen on the virion surface. The matrix protein (M) lines the internal surface of the virion envelope. The M2-2 regulatory factor is believed to have a role in regulating RNA synthesis.

Immunization studies and sero-epidemiological data have demonstrated that some antibodies recognising the G glycoprotein have neutralizing activity against RSV infection. The G glycoprotein was initially designated as the RSV attachment protein because antibodies specific to G inhibited the adsorption of virions to HeLa cells, whereas antibodies to the fusion (F) protein prevented fusion of the viral and cellular membranes, but not binding. The G glycoprotein binds to glycosaminoglycans on the cell surface and this may be important during the initial stage of the infectious cycle. However, recent findings suggest that the G glycoprotein may not be important for the infectious process in cell culture. A mutant virus, designated B1cp-52, has been isolated following multiple passages of the B1 strain of RSV subgroup B in Vero cells. The genome of this mutant lacks a large section of sequence encoding the majority of the G and SH proteins. This replicates efficiently in cell culture, however, its replicative efficiency was only moderate in cotton rats and very low in humans.

The mature glycosylated form of the RSV-G glycoprotein has an estimated M_r of 80-90 kD in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analysis of the nucleotide sequence of RSV-G mRNA showed it to be 918 nucleotides long with a single major open reading frame encoding a polypeptide chain of 289-299 amino acids, depending on the virus strain, with a M_r of approximately 32, 587. This finding suggested that over 50% of the molecular mass of the mature G glycoprotein is probably contributed by glycosylation. The 32.5 kD polypeptide precursor is co-translationally modified with high mannose N-linked sugars to generate an intermediate of 45 kD. After this step, the N-linked sugars are converted to complex types by the addition of O-linked sugar side chains achieving the mature 90 kD from the G glycoprotein in the trans-Golgi compartment. From there the G glycoprotein is transported to the cell surface. In the ectodomain of the G glycoprotein, serine and threonine residues are the acceptor sites for O-linked oligosaccharides. They are present in the sequence

at a high level, accounting for 30.6% of the total amino acid composition, compared with an average of 13% for most proteins. In addition, the G glycoprotein has an unusually high content of proline residues, 10.1% in comparison with an average of 5%. The numbers and locations of the serine and threonine residues vary from strain to strain with the average number in the ectodomain (and hence the number of O-linked glycosylation sites) of G being about 70. In contrast, the number of potential N-linked sugar sites is much lower, ranging from three in subgroup B viruses to between four and eight in subgroup A viruses (A2 and Long strains respectively). These features of amino acid composition and high O-linked sugar content are similar to those of mucins, a class of proteins produced and secreted by epithelial cells. The importance of the high sugar content is not yet known, however, it is postulated that the sugar might cover antibody epitopes allowing the virus to avoid recognition by the immune system.

The G glycoprotein is classified as a type II transmembrane glycoprotein. It is anchored into the membrane via an uncleaved hydrophobic signal-anchor sequence close to its N-terminus. The protein is comprised of three domains, a cytoplasmic domain beginning at amino acids 1-37, a membrane spanning sequence at amino acids 38-66 which serves as both signal peptide and transmembrane anchor, and an ectodomain at amino acids 67-298. The G ectodomain contains a central hydrophobic conserved region with four cysteine residues at position 173, 176, 182 and 186, which are highly conserved in all RSV strains. The variability seen in the G glycoproteins from different RSV strains is focused in the ectodomain which exhibits 56% diversity between subgroup A and B viruses, compared with 17% for the transmembrane and cytoplasmic domains. Analysis of escape mutants resistant to RSV monoclonal antibodies has also shown that most of the mutants had amino acid changes in variable regions of the G protein. Studies using monoclonal antibodies have identified three types of epitopes in the G molecule: (1) strain-specific or variable epitopes (2) subgroup-specific epitopes, and (3) conserved epitopes shared by strains from both A and B subgroups.

RSV synthesizes two mature forms of the G glycoprotein: a full-length anchored type II membrane form and a soluble form which is smaller and secreted into the medium. Analysis of the proteins synthesized from mutant cDNAs has established that the secreted form is generated by initiation at the second available AUG codon that is located near the middle of the transmembrane anchor domain. This results in the production of a protein with a shortened hydrophobic amino terminus, which enables the protein to pass through the

endoplasmic reticulum membrane. The G glycoprotein is then cleaved by proteolytic enzymes either between amino acid residues 65 and 66 or possibly between residues 74 and 75, and secreted. The soluble G glycoprotein possesses the same characteristics as the membrane-associated form of G; i.e., extensive glycosylation and reactivity with the anti-G monoclonal antibodies. The role of this protein is unknown, but it has been postulated that it could divert the immune response away from the infectious virions, or even mediate immunopathological mechanisms.

The changing of key antigenic determinants is a mechanism pathogens frequently use to escape the host immune response. Antigenic polymorphisms among RSV isolates were first reported in 1996 by a demonstration of small reciprocal difference in neutralization between the A2 and 18537 strains. By antigenic analysis using monoclonal antibodies specific to several RSV structural proteins, isolates of RSV can be divided into two distinct serological subgroups, A and B. During the annual epidemics of the past three decades, mixtures of strains from the two subgroups have been isolated, although isolates of subgroup A have been more prevalent than those of subgroup B.

Analysis using protease digestion and relative mobility on SDS-PAGE, in addition to reactivity with panels of mAbs, has demonstrated that antigenic variation can also be found in the F, G and P proteins. Furthermore particular variants are associated with either the A or B subgroups. Subsequent findings have clearly shown that the dimorphism is attributable primarily to variability of the attachment G glycoprotein. Sequencing studies have demonstrated that the G protein, particularly the extracellular domain, appears to show the highest level of divergence between the subgroups, with only 53% amino acid identity between the subgroup A compared to subgroup B isolates whereas the F proteins are relatively conserved with 90% similarity between subgroups. However, within the subgroups the G protein contains much less variation with 94% and 98% amino acid identity reported between strains A2 and Long of subgroup A, and between strains 18537 and 8/60 of subgroup B respectively.

Although a high level of sequence conservation has been reported, analysis of clinical isolates has demonstrated nine antigenic groups of the F protein within subgroup A and five within subgroup B. In a similar study, analysis using a panel of monoclonal antibodies directed against the F and G glycoproteins resulted in the identification of six variants in

subgroup A and three in subgroup B. PCR analysis and nucleotide sequencing of the SH genes together with restriction endonuclease mapping of the nucleocapsid protein genes of RSV isolates have discriminated five distinct lineages within subgroup A and two for subgroup B, based on the divergence of the SH and N genes. This work was extended to sequence the G gene of RSV isolates from subgroup A, and the result confirmed that five subgroups A lineages were identified. Multiple variants of RSV co-circulating during epidemics have been identified, and the variants found can be present in more than one epidemic season. An epidemiological study carried out in Birmingham, U.K. has demonstrated that group A RSV isolates predominated in eight epidemics during 11 years and each epidemic consisted of different genotypes. The relative prevalence of different genotypes was not consistent. Those found to predominate in one year declined in the next epidemic. Accordingly it will be appreciated that the development of agents that act specifically against RSV is difficult because of the variations that exist, or evolve, between viral strains.

Mutagenesis studies and the analysis of monoclonal antibody escape mutants have indicated that the G protein can sustain large deletions or multiple point mutations without loss of function. The driving force responsible for G protein heterogeneity is not known for certain. However it is currently thought that most of the diversity in the G protein and elsewhere in the genome has developed as a mechanism to avoid host immunity. The antigenic heterogeneity between RSV subgroups or even within a subgroup probably promotes the likelihood of re-infection despite the presence of pre-existing immunity to other, antigenically distinct RSV strains. However, the reason why the same degree of variation is not present in the F protein is unclear.

RSV infection is a major cause of admission to hospital for very young children. Epidemics occur throughout the world during the winter months in temperate regions, or during the rainy season in tropical regions. Over 50% of infants encounter RSV infection within their first two years of life and usually demonstrate upper respiratory tract symptoms. Infants under 6 months of age, with the highest incidence at 2 months, may develop more serious symptoms of lower respiratory tract infection (LRTI). This results in hospital admission of approximately 0.5-2% of infants during their first years of life. There is no accurate estimate for the overall death rate and different rates are found in different areas. However a survey of RSV infected children who required hospital treatment showed a mortality rate of 0.5-2.5%. A separate study carried out in the USA indicated that, between

1980-1996, RSV was responsible for an estimated 73,400 - 126,300 annual admissions to hospital or bronchiolitis and pneumonia among children under one year old and for 4,500 deaths annually. Modern intensive care techniques can help reduce the mortality rate of RSV disease. However, the mortality rate from RSV infection in infants with underlying illnesses (e.g. congenital heart disease, pulmonary hypertension or immunodeficiency) is likely to remain high. It will therefore be appreciated that there is a need to provide new or improved therapies for preventing or treating RSV infections.

During RSV infection, virus replication in the nasopharynx results in a large number of virions being released into the nasal secretions. RSV transmission via transfer of large droplets is a more important route than small-particle aerosols. Transmission occurs easily through close contact with infected persons and subsequent contact between the fingers and the nose or conjunctiva. Experimental RSV infection may be easily achieved by inoculating tissue culture-grown virus either into the nose or onto the conjunctiva.

With regard to RSV disease in infants, the incubation period from time of infection to onset of illness is about 4-5 days. Many different areas of the respiratory tract, from the nose to the lung, are infected by the virus. At the beginning of infection the virus replicates in the nasopharynx, and virus particles can be isolated from nasal secretions. In some infected infants, the virus shedding lasts for 3 weeks after initial hospitalisation. Many studies have reported some correlation between severity of disease, and the level of virus replication and duration of virus shedding.

The exact mechanism by which virus spreads from upper to lower respiratory tract is unclear. However it is assumed that it spreads via the respiratory tract epithelium, or through aspirated secretions. RSV can spread from cell to cell without emergence into the extracellular fluid. The onset of lower tract disease symptoms indicates that the virus has spread into the bronchi and bronchioles. It has been shown that even in a LRTI, RSV is still primarily localized in the superficial layers of the respiratory epithelium. There was no culturable virus found in blood samples of immunologically normal subjects although the presence of RSV antigens or RSV RNA may be detected in blood. However, in immunocompromised individuals, the virus can spread into other organs, such as kidney, liver, and myocardium.

Presently, there is no certain conclusion regarding what actually causes the RSV illness. Both the virus-mediated cytopathic effect and the anti-viral immune responses have been studied. RSV infection causes considerable damage to the important apparatus of respiratory tract, the epithelium and the bronchiolar cilia. Interfering with the removal of mucus and cell debris, this results in an accumulation of exudates in bronchioles and alveoli and causes obstruction of the small bronchioles, with either collapse or emphysema of the distal portion of the airway. Where pneumonia occurs, the interalveolar walls thicken due to mononuclear cell infiltration, and the alveolar spaces fill with fluid.

Many studies have concentrated on possible immunological mechanisms of RSV disease. The possible roles of both specific antibody and cellular immune responses have been investigated. Early studies suggested that the appearance of maternally derived RSV antibodies in infant sera coincided with the occurrence of severe RSV disease and suggested that immuno-pathologic mechanisms might be involved in RSV bronchiolitis. However, neither systemic (IgM and IgG) antibody, local (IgA) antibody nor local interferon responses show any correlation with severity of the illness in infants. In addition, there is much evidence to suggest that serum antibodies to RSV protect against rather than enhance RSV pathology. In B-cell depleted BALB/c mice RSV specific antibody is not important in terminating RSV replication, but does reduce illness in both primary RSV and repeat infections.

Similarly, it has been suggested that cell-mediated immune responses may play a role in the pathogenesis of RSV-induced disease. Passively transferred polyclonal memory T cells have been shown to clear persistent RSV infection in immunodeficient mice. However, intravenous transfer of RSV-specific cytotoxic T cells into previously irradiated RSV-infected mice resulted in enhanced pathology associated with accelerated clearance of virus from the lungs. Further studies in BALB/c mice have revealed that both CD4⁺ and CD8⁺ lymphocyte subsets are involved in termination of RSV replication after primary infection and in the pathologic process, but that CD8⁺ cells were more potent in both functions. In the absence of both T cells subsets, it has been suggested, therefore, that the host immune response rather than viral cytotoxic effect is the primary determinant of disease in mice, and a balance exists between the protective and disease-producing effect of CD8⁺ lymphocytes. Recently, several observations have demonstrated that the Th2 subset of CD4⁺ T lymphocytes is associated with RSV pathogenesis in human infants. To date, it is not clear what exactly causes pathology in

RSV disease in human infants, however, it may be that immunologic mechanisms also play an important role here.

Host immune responses play a primary role in recovery from RSV infection and resistance to reinfection. This is consistent with the observation that immunodeficient children fail to clear RSV and have prolonged virus shedding for many months rather than the typical interval of 1-3 weeks. In addition, it has been found that patients treated with immunosuppressive drugs, such as leukaemia patients or bone marrow transplant recipients, have a very high incidence of RSV infection which may subsequently lead to serious disease and death. The immune response to RSV disease results in the production of secretory and serum antibodies, and MCH class I-restricted CTLs all of which have been implicated in the control of virus infection. Exactly which component of the immune response is the most important in protecting against infection has been the subject of much research.

Due to the high level of hospitalisation and morbidity associated with RSV infection (see above), the WHO has given a high priority to the development of effective treatments for RSV and in particular an effective vaccination strategy.

A formalin-inactivated aluminium-precipitated RSV vaccine was the first strategy to be developed and tested in infants and children three decades ago. However, not only did this vaccine fail to protect against natural RSV infection, but on rechallenge with RSV it actually enhanced disease potentiation resulting in an increased hospitalisation rate for lower respiratory tract pathology and a high rate of mortality.

After the failure of formalin-inactivated RSV, further attempts to develop a vaccine concentrated on the administration of live virus. Unattenuated virus, derived by ten passages of a clinical isolate of RSV in diploid lung fibroblasts, was administered subcutaneously. This vaccine induced good immune responses, including neutralizing antibodies, in sero-negative children. However, it failed to protect in very young infants with transplacentally acquired antibody, and there was no difference in the incidence of natural infection in infants and children in vaccine and placebo groups.

Two forms of attenuated RSV vaccines for administration intranasally have been developed (cold-passaged (*cp*) viruses and temperature-sensitive (*ts*) mutants). However live

vaccine candidates which were avirulent, but immunogenic and protective in adults, were initially found to be unacceptable in infants due either to residual virulence, over-attenuation or genetic instability. The live attenuated vaccine approach has potential advantages in that the vaccine can be administered intranasally and so should induce an immune response that will mimic natural infection, raising a balanced humoral, cell-mediated, and secretory immune response. It should be immunogenic in the presence of transplacental antibodies, and not be likely to induce vaccine-enhanced illness. In contrast, a potential disadvantage may be an insufficient attenuation in seronegative infants whose immune response may not be competent for protection against natural infection. It is also unclear how effective such a single strain vaccine would be in protecting infants against the diverse antigenic variants of RSV which circulate in the community.

An alternative approach has been the development of a subunit vaccine. This required the identification of the RSV antigens that are the important targets for protective immune responses. A study of the protective efficacy of purified proteins, (the fusion glycoprotein (F), the large glycoprotein (G), the phosphoprotein (P), and the 22k protein (M2), of RSV strain A2) as well as purified fusion proteins thereof demonstrated that all of the proteins were immunogenic and to a variable degree were able to raise neutralizing antibodies. However such vaccines are less than ideal because of the problems of strain variation/evolution discussed above.

A chimaeric RSV-FG candidate vaccine comprising an immunoaffinity-purified, baculovirus-expressed chimaeric glycoprotein containing the ectodomains of RSV F and G has also been tried as a strategy to induce protective immune response against RSV infection. Intramuscular delivery of this vaccine showed protective activity in the lower respiratory tracts but not in the upper respiratory tract of rodents. Pulmonary pathologic enhancement after wild-type RSV challenge of cotton rats previously immunized with the chimaeric RSV-FG remains unclear as it was observed in some studies but not others.

In 1997 a new recombinant vaccine, designated BBG2Na, was developed in which an RSV G protein fragment (G2Na), corresponding to amino acid residues 130-230 from an RSV subgroup A strain virus, but containing a known epitope conserved in both subgroup A and B of RSV, was fused with the albumin-binding region (BB) of streptococcal protein G. This vaccine has been examined in BALB/c mice and cotton rats, and long-term protection of the

lower respiratory tract against a challenge with RSV subgroup A has been demonstrated by intramuscular injection. However, upper respiratory tract protection was poor in mice and absent in cotton rats. When challenged with a representative RSV subgroup B strain, both mice and cotton rats showed protective immune responses in the lungs after immunization with BBG2Na although the cross-protection was less effective than homologous protection. Changing the BBG2Na vaccine delivery route to intraperitoneal administration induced an immune response sufficient for upper respiratory tract protection in mice, but still not for cotton rats. These results suggest that choice of immunization route is important and that nasal tract protection is species-dependent. Passive Immunization with anti-BBG2Na antibody resulted in both lung protection against RSV subgroup A challenge and virus clearance. However no protection was observed in the mouse nasal cavity.

The introduction of RSV genes into vaccinia virus offers another possibility for vaccination. Recombinant vaccinia viruses encoding all of the RSV specific proteins, except for the polymerase (L), have been studied individually for their abilities to induce resistance to RSV infection in BALB/c mice. Vac-F and Vac-G (recombinant vaccinia virus expressing RSV F and RSV G glycoprotein respectively) were the most protective vaccines inducing neutralizing antibodies and protecting mice against RSV challenge. Mice immunized with Vac-M₂ or Vac-N were also partially resistant to RSV infection, but in this case resistance was probably mediated by cytotoxic T-lymphocytes, as no neutralizing antibodies were produced. Although both vaccinia recombinants induced neutralizing antibody levels which surpassed the level required to protect the lung of cotton rats against RSV infection, sero-negative chimpanzees immunized intradermally with Vac-F and Vac-G produced only low levels of neutralizing antibody, leading to incomplete protection of the lower respiratory tract and no protection of the upper respiratory tract against RSV challenge. Due to concerns about safety of live recombinant vaccinia viruses in young infants together with their lack of immunogenicity in chimpanzees, these reagents are no longer considered to be candidate vaccines.

Enteric immunization with wild-type adenovirus in humans has been shown to be safe and to result in specific immune responses in the respiratory tract. Thus adenoviruses are alternative vectors that have been used to produce recombinant RSV vaccines. A wild type strain of adenovirus (Ad5) has been used to construct an adenovirus recombinant vaccine expressing RSV F (Ad5F). This vaccine produced complete or partial protection in cotton rats

when immunized via the intranasal and enteric routes respectively. As intranasal administration of this virus into humans may cause respiratory illness due to the adenovirus itself, an attenuated adenovirus would be a preferable vector. Subsequently, therefore, vaccine strains of Ad4, Ad7, as well as Ad5 were used for the construction of recombinant vaccines expressing F or G, or F and G together. Intratracheal immunization of dogs with Ad7F induced immune responses with moderate titres of RSV-neutralizing antibodies. The antibody level was even higher after booster immunization with Ad4F. Three two-dose vaccination regimens, Ad4F, Ad5F, Ad7G/Ad4G, and Ad7FG/Ad4FG, were also evaluated for immunogenicity and protective efficacy in dogs. It seemed that the adenovirus recombinants encoding F alone were more immunogenic than recombinants expressing G alone or F and G together. However, when tested in a chimpanzee sequential oral administration of Ad7F, Ad4F, and Ad5F, only induced a low level of antibody response to RSV.

Recently, a recombinant bacteriophage has been constructed by inserting a short stretch of nucleotides encoding the G glycoprotein amino acids 173-187, which contain a known protective epitope, into the 'gene III' protein gene of fd bacteriophage. The G protein peptide was therefore expressed and fused to pIII coat protein on the surface of phage particle. This vaccine elicited a strong immune response in mice, with a high level of circulating RSV-specific antibodies, leading to complete resistance against RSV infection on challenge.

Since efforts to produce an effective vaccine have been unsuccessful (see above), the development of an effective antiviral agent for RSV infection has been pursued as an alternative strategy for prophylaxis as well as for treatment. Ribavirin (1- β -D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) is a virostatic synthetic nucleoside analogue resembling guanosine that is active against the replication of a large number of both RNA and DNA viruses. After *in vivo* administration, ribavirin is phosphorylated intracellularly to ribavirin triphosphate, which is a highly active metabolite that inhibits viral replication. It is subsequently catabolised to triazole carboxamide, an inactive metabolite which is excreted in the urine.

Ribavirin has been administered via the oral and parenteral routes, or directly into the respiratory tract in the form of a small particle aerosol. Owing to its toxic effects on the liver and bone marrow when administered orally, and because the replication site of RSV is the respiratory tract, most recent clinical trials have used aerosolised ribavirin. Several studies

have indicated that the delivery of ribavirin as an aerosol for two to five days results in a decrease in the severity of signs and symptoms of RSV infection. An effective dose of aerosolised ribavirin in cotton rats is about 5 mg of ribavirin per kilogram of body weight per day. In human infants suffering from RSV-induced bronchiolitis, an estimated daily dose of approximately 10 mg of ribavirin per kilogram of body weight has been recommended. Due to concerns over the expense of delivery and possible residual toxicity of aerosolised ribavirin to caregivers and families, the use of higher doses of ribavirin administered over shorter period has been evaluated. This study showed a similar result to those that used standard-dose therapy.

Although the studies described above indicate that the administration of ribavirin is an effective treatment for RSV infection, some subsequent studies have shown contradictory results. A retrospective study to evaluate ribavirin therapy for lower respiratory tract RSV infection failed to reveal significant differences in length of stay, mortality rate, and duration of oxygen therapy between treated and untreated patients. A double-blind placebo-controlled study of patients with lower respiratory tract RSV disease also failed to demonstrate a positive effect. In addition, in a further study the use of ribavirin as a therapy was actually associated with prolongation of hospitalisation and requirement for respiratory intervention. The American Academy of Paediatrics have reviewed ribavirin treatment for RSV infection and have recommended that it should only be considered for use in high risk children with severe RSV illness.

Interferon- α (IFN- α) is produced and secreted into the extracellular fluid by many cell types in response to viral infections and may induce resistance against viral replication in host cells. Trials with IFN- α have produced variable results and it has been concluded that IFN- α -2a treatment provides no great benefit in the treatment of RSV disease.

Therapeutic antibodies have also been considered for the treatment of RSV infections. Epidemiological studies have provided three lines of evidence supporting the use of RSV neutralizing antibodies for immunoprophylaxis and immunotherapy of RSV infection. They are (1) a lower incidence of RSV infection in infants born to mothers with high levels of RSV-neutralizing antibody. (2) Infants with a high titre of maternal RSV-neutralizing antibody

remain free of RSV infection for a longer period than those who have lower titres and (3) their re-infections with RSV tend to be less severe than the primary infection.

Human immunoglobulin specific for RSV purified from plasma donors has been evaluated for the prevention and treatment of RSV infection. It has been demonstrated that, following parenteral administration, high-titre RSV human immunoglobulin is effective for both prophylaxis and treatment of RSV infection in cotton rats and owl monkeys, without potentiation of pulmonary pathology. These observations provided the experimental basis for a subsequent clinical trial in humans. The therapeutic study of human intravenous immunoglobulin (IVIG) containing substantial levels of RSV neutralizing antibody in human infants and children with RSV pneumonia or bronchiolitis resulted in a marked reduction in nasal virus shedding, and an improvement in transcutaneous oximetry reading without disease exacerbation. However, no reduction in the duration of hospitalisation was observed. As RSV initiates infection in the nasopharynx, in theory the direct application of antibodies to the mucosal surface could provide better protection than systematic administration. When administered directly into the lungs of cotton rats, human IgG with a high titre of RSV neutralizing antibody was approximately 160 times more effective therapeutically than IgG inoculated parenterally, suggesting that smaller quantities of the antibody could be used for the same therapeutic effect. In contrast, other workers found that aerosolised human IgG did not provide any relief from RSV illness. This prompted concern over the preparation of standard human immunoglobulin with reproducibly adequate RSV neutralizing antibody titres, leading to the development of more reliable techniques for the isolation of RSV antibodies from pooled human plasma.

Respiratory Syncytial Virus Immune Globulin Intravenous (Human) (RSV-IGIV) or RespiGamTM is a polyclonal human immunoglobulin G enriched in neutralizing antibodies to RSV that is prepared from pooled human plasma donors selected for high serum titre of RSV-neutralizing antibodies. RespiGamTM was the first antibody preparation demonstrating clinical benefit for prevention of severe RSV disease in high-risk infants and children, and was licensed by the FDA in 1996. The use of RSV-IGIV compared to a placebo control, was subsequently investigated in premature infants with BPD by the PREVENT group in 1997. Patients receiving high dose RSV-IGIV had a 41% reduction in the probability of hospitalisation due to RSV, and 53% reduction in the total length of stay if hospitalised. Several disadvantages associated with the use of RSV-IGIV were raised, e.g., the potential for

recipients to contact blood-born pathogens; obtaining and securing intravenous access; interference with routine vaccinations, and the cost. Based on the above data and problematic drawbacks, the FDA approved RSV-IGIV (RespiGamTM) for use in the prevention of RSV infection in premature infants and children younger than 2 years of age with BPD, but it is not recommended for patients with CHD.

Attempts to improve the efficacy of antibody-mediated RSV prophylaxis and treatment have focused on the source of the antibody. Due to their high specificity and specific activity monoclonal antibodies (mAbs) are an attractive alternative to pooled human immunoglobulin for RSV prophylaxis and therapy. A study carried out in cotton rats demonstrated that intraperitoneal, passive immunization with mouse neutralizing mAbs specific for the RSV glycoproteins F and G resulted in a significant reduction of viral titre in lung tissue after intranasal challenge with virus. However no sign of enhanced pulmonary pathology was seen. HNK-20, a purified mouse monoclonal IgA antibody specific for the F glycoprotein has also been evaluated. Passive nasal instillation of the antibody was no more effective in decreasing the RSV titre in the lungs of mice than an F-specific IgG mAb, and it was less effective than systemically administered IgG mAb in prophylaxis. However, a controlled trial in which several hundred infants at risk of severe RSV disease received nasal instillation of HNK-20 did show a beneficial trend of decreasing hospitalisation among infants under 4 months of age.

The use of rodent monoclonal antibodies for human therapy is undesirable and it is preferred that humanised monoclonal antibodies are produced by genetic engineering for human treatment. Two humanised monoclonal antibodies have been evaluated in the clinic RSHZ19 or SB209763 (SmithKline Beecham Pharmaceutical, Conshohocken, PA), a human IgG1_K antibody recognizing an F protein neutralizing epitope, was tested in a multicentre double blind, randomised, placebo-controlled, international study in preterm infants or children with BPD or CHD. The result showed that RSHZ19 was not efficacious in reducing hospitalisation for RSV-induced disease. The second mAb is Palivizumab (aka MEDI-493 or SynagisTM), which is also a humanised IgG1_K antibody specific for the F glycoprotein, is the first and only monoclonal antibody licensed for an infectious disease indication in the USA. A clinical trial of the antibody was carried out on high-risk infants and children by the Impact RSV study group during 1996-1997. In contrast to the experience with RSHZ19, this randomised, double-blind, placebo controlled study indicated that a dose of 15 mg/kg of

antibody intramuscularly administered each month to patients produced a 55% reduction in hospital admissions for RSV illness compared to the placebo control group. Recently, a comparative study of these two humanized antibodies demonstrated that Palivizumab was 4-5 fold more effective than RSHZ19 in terms of antigen binding, RSV neutralization, and fusion inhibition which might explain the difference in their relative *in vivo* performances. Palivizumab was approved by the FDA in 1998 for prophylaxis against severe RSV disease in premature infants and children with chronic lung disease (CLD). However, to date no antibody is approved as a therapeutic agent for RSV disease.

It will be appreciated, in the light of the disadvantages associated with most prior art vaccines and therapeutic agents, that there is a need to provide new and improved agents for preventing the development of RSV infections and for treating existing RSV infections. Given that Synagis™ (a humanised IgG1K antibody specific for the F glycoprotein) appears to be one of the most successful developments to date, one object of the present invention has been to develop new and improved monoclonal antibodies that may be useful in the prevention or treatment of RSV infections.

According to a first aspect of the present invention, there is provided an antibody, or a functional derivative thereof, against the G glycoprotein of Respiratory Syncytial Virus characterised in that a Variable Region comprises:

(i) a first domain containing at least one peptide sequence selected from:

- (a) Arg Ser Ser Gln Asn Ile Val His Ser Asp Gly Asn Thr Tyr Leu Glu (SEQ ID No. 1); or
- (b) Lys Val Ser Asn Arg Phe Ser (SEQ ID No. 2); or
- (c) Phe Gln Gly Ser His Ile Pro Trp Thr (SEQ ID No. 3)

derivable from an antibody V_L region; and

(ii) a second domain containing at least one peptide sequence selected from:

- (d) Asp Tyr Ala Met His (SEQ ID No. 4); or
- (e) Val Ile Ser Thr Tyr Tyr Gly Asn Pro Asn Tyr Asn Gln Lys Phe (SEQ ID No. 5); or
- (f) Ser Asp Met Ile Thr Ala Gly Gly Gly Tyr Ala Met Asp Tyr (SEQ ID No. 6)

derivable from an antibody V_H region.

According to a second aspect of the present invention, there is provided a composition comprising an antibody, or functional derivative thereof, according to the first aspect of the invention and a pharmaceutically acceptable vehicle for use as a medicament.

According to a third aspect of the present invention, there is provided an antibody, or functional derivative thereof, according to the first aspect of the invention for the manufacture of a medicament for the prevention or treatment of Respiratory Syncytial Virus infections.

According to a fourth aspect of the present invention, there is provided a method for the prevention or treatment of Respiratory Syncytial Virus infections comprising administering to a person or animal in need of such treatment a therapeutically effective amount of an antibody, or functional derivative thereof, according to the first aspect of the invention.

Antibodies or immunoglobulins contain two characteristic functions: (a) binding specificity to an antigenic determinant and (b) participation in effector functions: e.g., complement activation, stimulation of phagocytosis by macrophages, and inducing mast cell degranulation. In their simplest form, immunoglobulin proteins are Y-shaped molecules usually exemplified by the γ -immunoglobulin (IgG) class of antibodies. The molecule consists of four polypeptide chains two identical heavy (H) chains and two identical (L) chains of approximately 50kD and 25kD each respectively. Each light chain is bound to a heavy chain (H-L) by disulphide and non-covalent bonds. Two identical H-L chain combinations are linked to each other by similar non-covalent and disulphide bonds between the two H chains to form the basic four chain immunoglobulin structure (H-L)₂.

Light chain immunoglobulins are made up of one V-domain (V_L) and one constant domain (C_L) whereas heavy chains consist of one V-domain and, depending on H chain isotype, three or four C-domains (C_{H1}, C_{H2}, C_{H3} and C_{H4}).

At the N-terminal region of each light or heavy chain is a variable (V) domain that varies greatly in sequence, and is responsible for specific binding to antigen. Antibody specificity for antigen is actually determined by amino acid sequences within the V-regions known as hypervariable loops or Complementarity Determining Regions (CDRs). Each H and L chain V regions possess 3 such CDRs, and it is the combination of all 6 that forms the

antibody's antigen binding site. The remaining V-region amino acids which exhibit less variation and which support the hypervariable loops are called frameworks regions (FRs).

In the light of the above, it will therefore be appreciated that preferred antibodies, or a functional derivatives thereof, comprise:

- (a) a first domain comprising a V_L region with all three of the CDRs of amino acid sequence ID No. 1, 2 and 3; and
- (b) a second domain comprising a V_H region with all three of the CDRs of amino acid sequence ID No. 4, 5 and 6;

The regions beyond the variable domains (C-domains) are relatively constant in sequence. It will be appreciated that the characterising feature of antibodies according to the invention is the V_H and V_L domains. It will be further appreciated that the precise nature of the C_H and C_L domains is not, on the whole, critical to the invention. In fact preferred antibodies according to the invention may have very different C_H and C_L domains. Furthermore, as discussed more fully below, preferred antibody functional derivatives may comprise the Variable domains without a C-domain (e.g. scFV antibodies).

The inventors have found that antibodies, or functional derivatives thereof, according to the first aspect of the invention have surprising efficacy for preventing the development of RSV infections and for treating existing RSV infections.

Although we do not wish to be bound by any hypothesis, we believe that antibodies according to the first aspect of the invention are useful because they are raised against the G Glycoprotein and more particularly to an epitope on the G glycoprotein that the inventors believe represents a good target for RSV neutralisation.

In animal models, both active and passive immunizations have demonstrated that antibodies to the RSV G glycoprotein confer protection that is sub-group specific, whereas anti-F antibodies provide cross-reactive protection. Conventional wisdom may therefore suggest that F is a more attractive target for vaccine development and immunoprophylaxis. However prior art antibodies specific for F appear less effective in human infants than in animal models (see above). Even Palivizumab, the best anti-F mAb tested in clinical trials to

date, only reduces hospitalisation rates by half and does not give full protection even though it cross- reacts with both A and B strains of RSV.

In tissue culture systems, neutralizing anti-G mAbs generally have much weaker activity than neutralizing anti-F mAbs. This weak *in vitro* neutralizing activity is therefore a factor that has persuaded researchers to focus on the development of anti-F mAbs for immunoprophylaxis and immunotherapy of RSV. Furthermore the goal of making a therapeutic anti-G mAb protective against all RSV strains is rendered even more difficult because of the high antigenic variability of the extracellular domain of the protein. Restriction mapping and nucleotide sequencing have revealed that the G gene sequences of isolates within the RSV subgroup A differ by as much as 20%. Up to 9% variability in the gene has also been shown in subgroup B isolates.

Despite this prejudice against anti-G mAbs, the inventors decided to investigate such antibodies in more detail.

They have found, to their surprise, that anti-G antibodies according to the invention are effective for preventing or treating RSV infections and in particular are far more effective *in vivo* than any *in vitro* data may suggest. Again, without being bound by any hypothesis, the inventors believe that antibodies according to the invention are effective against a broad spectrum of RSV strains because they are raised against conserved regions of the G-glycoprotein. In particular the inventors believe that the antibodies of the invention may bind to the conserved sequence amino acids 174-187 of the G Glycoprotein. Antibodies have previously been raised against amino acids 174-187. For instance, Trudel et al. (1991) raised an antibody against peptide 174-187 from the G glycoprotein and found it had some activity for neutralising RSV. However their antibody is unrelated to antibodies according to the present invention. In particular the Trudel antibodies do not have CDRs or whole VL or VH regions with the same sequences as the antibodies, or functional derivatives thereof, according to the invention. Nothing in Trudel et al. (1991) or other known prior art suggests that antibodies as defined herein will have such efficacy (as illustrated in the Examples) for preventing or treating RSV infections.

The antibodies, and functional derivatives thereof, according to the invention were developed following work conducted by the inventors on a murine antibody called 1C2. This

antibody has been referred to in a paper published by Morgan *et al.* [(1987) *Journal of Genetic Virology* 68, 2781-2788]. However, the binding specificity and amino acid sequence of IC2 have never been made available to the public. Furthermore, initial *in vitro* tests suggested that IC2 had poor efficacy for RSV neutralisation; IC2 was therefore not investigated any further; and the IC2 hybridoma stored in the applicant's private storage facilities. Several years after IC2 was first developed, the inventors were investigating a phage display for anti-G glycoprotein activity. Part of their investigation included the use of a bank of antibodies (including IC2) that they also believed to have anti-G glycoprotein activity. The inventors discovered, to their surprise, that IC2 appeared to bind avidly to a peptide corresponding to amino acids 172 - 187 of the G-glycoprotein. Furthermore it appeared to have very good RSV neutralising activity *in vivo* (see Example 1).

This investigation led the inventors to believe that IC2 and functional derivatives thereof, may be useful in the prevention or treatment of RSV infections. They therefore tested the efficacy of the antibody against RSV; further characterised the V_H and V_L domains of the antibody; and also developed humanised versions of IC2. Humanised forms of antibodies, and functional derivatives thereof, represent preferred antibodies and functional derivatives according to the invention. Most preferred humanised forms are discussed in more detail below and in the Examples.

Preferred antibodies, and functional derivatives thereof, according to the first aspect of the invention may have the V_L (first domain) and V_H (second domain) domains identified in Figures 5 and 4 respectively or are close derivatives thereof. A derivative thereof may have 75% sequence identity, more preferably 90% sequence identity and most preferably has at least 95% sequence identity. It will be appreciated that most sequence variation may occur in the framework regions (FRs) whereas the sequence of the CDRs of the antibodies, and functional derivatives thereof, according to the first aspect of the invention is most conserved.

A number of preferred embodiments of the first aspect of the invention relate to molecules with both Variable and Constant domains. However it will be appreciated that antibody fragments (e.g. scFV antibodies) are also encompassed by the invention that comprise essentially the Variable region of an antibody without any Constant region. Such antibody fragments may comprise CDRs of SEQ ID No.s 1 - 6.

An scFv antibody fragment according to the invention may comprise the whole of the V_H and V_L domains of IC2. The V_H and V_L domains may be separated by a suitable linker peptide. A preferred scFv antibody is described in the Examples and its sequence illustrated in Figure 3.

mAbs generated in one species are known to have several serious drawbacks when used to treat a different species. For instance when murine antibodies are used in humans they tend to have a short circulating half-life in serum and are recognised as foreign proteins by the patient being treated. This leads to the development of an unwanted human anti-mouse (or rat) antibody (HAMA) response. This is particularly troublesome when frequent administrations of the mAb are required as it can enhance the clearance of the mAb, block its therapeutic effect, and induce hypersensitivity reactions. In addition, only one isotype of immunoglobulin is produced from one hybridoma cell line. As not all of the isotypes are able to trigger the appropriate system of complement and Fc receptors that may be required to achieve the desired therapeutic effect this may result in a poor therapeutic outcome. These factors limit the use of mouse monoclonal antibodies in human therapy and have prompted the development of antibody engineering technology to generate humanised antibodies. Accordingly preferred monoclonal antibodies (of non-human source) for use in human therapy are humanised (as described in more detail below and in the Examples)

Monoclonal antibodies are generated by the hybridoma technique which usually involves the generation of no-human mAbs. The technique enables rodent monoclonal antibodies with almost any specificity to be produced. Accordingly the inventors used such a technique to develop the IC2 antibody against RSV. Although the inventors have established that IC2 antibody is useful therapeutically, it will be appreciated that such antibodies are not ideal therapeutic agents in humans (as suggested above). Ideally, human monoclonal antibodies would be the preferred choice for therapeutic applications. However, the generation of human mAbs using conventional cell fusion techniques has not to date been very successful. The problem of humanisation has been addressed by recent, rapid advances in antibody engineering which have made it possible to build "human-like or humanised antibodies." Such engineering using V region sequences from non-human (e.g. rodent) mAbs and C region (and ideally FRs from V region) sequences from human antibodies. The resulting 'engineered' mAbs are less immunogenic in humans than the rodent mAbs from which they were derived and so are better suited for clinical use.

Accordingly preferred antibodies, or functional derivatives thereof, according to the invention have CDRs of SEQ ID No. 1- 6 (e.g. derived from the murine mAb 1C2) and other parts of the antibody (C domains etc) derivable from human antibodies.

Humanised antibodies may be chimaeric monoclonal antibodies, in which, using recombinant DNA technology, rodent immunoglobulin constant regions are replaced by the constant regions of human antibodies. The chimaeric H chain and L chain genes may then be cloned into expression vectors containing suitable regulatory elements and induced into mammalian cells in order to produce fully glycosylated antibodies. By choosing an appropriate human H chain C region gene for this process, the biological activity of the antibody (e.g., whether or not it has the potential to activate complement) may be pre-determined. Such chimaeric antibodies are superior to mouse monoclonal antibodies in that their ability to activate effector functions can be tailored for a specific therapeutic application, and the anti-globulin response they induce is reduced.

Such chimaeric molecules may be used to treat RSV according to the present invention. RT-PCR may be used to isolate the V_H and V_L genes from preferred mAbs, cloned and used to construct a chimaeric version of the mAb possessing human domains (e.g. human_K light chain and human γ 1 heavy constant regions)

Preferred chimaeric versions possess longer circulating half-life and/or may have a substantially reduced ability to induce the antiglobulin response compared to non-human equivalents.

Examples of such chimaeric molecules, which represent preferred antibodies according to the present invention, are given in Example 4 and their efficacy illustrated in Example 5.

Further humanisation of antibodies may involve CDR-grafting or reshaping of antibodies. Such antibodies are produced by transplanting the heavy and light chain CDRs of a rodent mAb (which form the antibody's antigen binding site) into the corresponding framework regions of a human antibody. Most preferred antibodies according to the present

invention comprise CDRs of SEQ ID No. 1- 6 inserted into a suitable human immunoglobulin.

Chimaeric and CDR-grafted antibodies may comprise the human immunoglobulin IgG and in particular IgG1. Preferably the light chain is a human kappa light chain and the heavy chain is a human γ 1 heavy chain.

Preferred antibodies, and functional derivatives thereof, according to the invention are able to elicit complement fixation. Immunoglobulins are classified as glycoproteins due to the N-linked polysaccharide present on the heavy chain constant region. The polysaccharide plays an important role in a variety of interactions with other serum proteins, cells and tissues, invoking responses such as complement fixation and the recruitment of cytotoxic effector cells. Accordingly it is preferred that antibodies according to the present invention retain amino acids in the C_H and C_L domains which are capable of glycosylation. For instance, it is preferred that the C_H2 domain N-linked glycosylation site is maintained in the antibody because the absence of sugars at this site may be associated with defective complement activation and Fc γ -R binding. A preferred chimaeric molecule capable of fixing complement is the glycosylated chimaeric molecule described in Examples 4 and 5.

The inventors have found that antibodies according to the present invention are surprisingly effective against a broad spectrum of RSV from subgroup A.

The antibodies, or functional derivatives thereof, may be used in a number of ways. For instance, systemic administration may be required in which case the antibodies or derivatives thereof may be contained within a composition which may, for example, be ingested orally in the form of a tablet, capsule or liquid. Alternatively the antibodies, or derivatives thereof, are administered by injection into the blood stream. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion).

It is most preferred that the antibodies, or derivatives thereof, are administered by inhalation. In this case they may be formulated in liquid or powder form for administration by conventional inhalation devices. The antibodies may be delivered to the lungs as small particle aerosols.

It will be appreciated that the required amount of the antibodies, or derivatives thereof, is determined by biological activity and bioavailability which in turn depends on the mode of administration, the physicochemical properties of the antibodies, or derivatives thereof, and whether the antibodies, or derivatives thereof, are being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the abovementioned factors and particularly the half-life of the antibodies, or derivatives thereof, within the target tissue or subject being treated.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials etc), may be used to establish specific formulations of the antibodies, or derivatives thereof, and precise therapeutic regimes (such as daily doses of the compounds and the frequency of administration).

Generally, a daily dose of between 0.01µg/kg of body weight and 1.0g/kg of body weight of the antibodies, or derivatives thereof, may be used for the treatment of RSV; more preferably the daily dose is between 0.01mg/kg of body weight and 100mg/kg of body weight

Daily doses may be given as a single administration (e.g. a single daily injection or a single dose from an inhaler). Alternatively the antibodies, or derivatives thereof, may require administration twice or more times during a day.

It will be appreciated from the Examples that molecular biology techniques were employed to characterise IC2 and to develop functional derivatives thereof. The genetic constructs developed in this work represent an important feature of the present invention. Therefore, according to a fifth aspect of the present invention, there is provided a DNA molecule coding an antibody Light Chain gene, or functional derivative thereof, characterised in that the DNA molecule comprises at least one nucleotide sequence selected from the group comprising:

- (a) AGA TCT AGT CAG AAC ATT GTA CAT AGT GAT GGA AAC ACC TAT
TTA GAG (SEQ ID NO .7); or
- (b) AAA GTT TCC AAC CGA TTT TCT (SEQ ID NO. 8); or
- (c) TTT CAA GGT TCA CAT ATT CCG TGG ACG (SEQ ID NO .9).

It is preferred that the DNA molecule of the fifth aspect of the invention comprises each of the nucleotide sequences 7 - 9 and most preferred that the DNA molecule codes the light chain shown in Figure 5.

According to a sixth aspect of the present invention, there is provided a DNA molecule coding an antibody Heavy Chain gene, or functional derivative thereof, characterised in that the DNA molecule comprises at least one nucleotide sequence selected from the group comprising:

- (a) GAT TAT GCT ATG CAC (SEQ ID NO .10); or
- (b) GTT ATT AGT ACT TAC TAT GGT AAT CCA AAT TAC AAC CAG AAG TTT (SEQ ID NO .11); or
- (c) TCG GAT ATG ATT ACG GCC GGC GGC GGG TAT GCT ATG GAC TAC (SEQ ID NO .12).

It is preferred that the DNA molecule of the sixth aspect of the invention comprises each of the nucleotide sequences 10 - 12 and most preferred that the DNA molecule codes the heavy chain shown in Figure 4.

It will also be appreciated that gene constructs coding the VH and VL regions of antibodies, or functional derivatives thereof represent important molecules commercially. Such constructs encode preferred functional derivatives of the first aspect of the invention (e.g. scFV fragments) and also represent good starting points for developing humanised forms of the antibodies. Therefore according to a seventh aspect of the invention there is provided a DNA molecule comprising a DNA molecule according to the fifth aspect of the invention linked, in-frame, to a DNA molecule according to the sixth aspect of the invention.

It will be appreciated that the DNA molecules according to the fifth, sixth and seventh aspect of the invention may be contained within gene constructs with additional function. For instance, the construct may be an expression cassette comprising a promoter operatively linked to the DNA molecule. The cassette may comprise further regulatory elements that assist in the expression of the peptides/proteins encoded by the DNA molecules.

Another important feature of the invention is the development of expression vectors that may be used to transform cells and drive expression of peptide and proteins that fall with

the scope of the invention. Therefore according to an eighth aspect of the invention there is provided an expression vector comprising DNA molecules according to the fifth - seventh aspect of the invention.

According to a ninth aspect of the invention there is provided a cell transformed with a DNA molecule according to the fifth, sixth or seventh aspect of the invention or a vector according to the eighth aspect of the invention.

Preferred DNA molecules, constructs, expression cassettes, vectors and cells are described in more detail in the Examples.

The present invention will now be described, by way of example, with reference to the accompanying drawing, in which:

Figure 1 illustrates the immunoprophylactic effect of the mouse monoclonal antibody (1C2) on RSV infection in BALB/c mice as described in example 1;

Figure 2 summarises the cloning strategy used to isolate the monoclonal antibody 1C2V_H and V_L [with reference to Example 2];

Figure 3 illustrates the DNA sequence (SEQ ID No. 44) and the theoretical amino acid sequence translation (SEQ ID No. 45) of the 1C2 scFv antibody (p530) [as identified in Example 2];

Figure 4 illustrates the DNA sequence (SEQ ID No. 46) and the theoretical amino acid sequence translation (SEQ ID No. 47) of the 1C2 V_H region of the heavy chain gene [as identified in Example 2];

Figure 5 illustrates the DNA sequence (SEQ ID No. 48) and the theoretical amino acid sequence translation (SEQ ID No. 49) of the 1C2 V_L regions of the light chain gene [as identified in Example 2];

Figure 6 shows the results of a competitive ELISA illustrating the binding of 1C2 scFv phage antibody (p530) to BSA-G antigen in the presence of original 1C2 mAb and anti-Adenovirus mAb [with reference to Example 3];

Figure 7 enlists the nucleotide and amino acid sequences of the chimaeric 1C2 heavy chains carried by M13mp19 clone M609 (glycosylated) (SEQ ID No. 50 and 52, respectively) and M610 (aglycosylated) (SEQ ID No. 51 and 53, respectively) [with reference to Example 4];

Figure 8 enlists the nucleotide and amino acid sequences of the chimaeric 1C2 kappa light chain (SEQ ID No. 54 and 55, respectively) carried by pEE12 plasmid p533 [with reference to Example 4]; and

Figure 9 illustrates the immunoprophylactic effect of mouse and chimaeric 1C2 anti-G monoclonal antibodies on RSV infection in BALB/c mice showing the geometric mean quantity of RSV recovered from the lungs of groups of 6 mice 4 days post intranasal challenge with virus. 24 hours prior challenge the mice were given a single intravenous dose of the indicated anti-RSV G or control antibody, or PBS. The error bars indicate the \pm 1 S.D. range of the means [with reference to Example 5].

EXAMPLE 1

The monoclonal antibody 1C2 is known to bind to the RSV G glycoprotein and was previously found to have poor *in vitro* RSV neutralisation properties relative to mAbs specific for the RSV F glycoprotein. Therefore, its therapeutic and/or prophylactic potential has previously been discounted.

Despite this poor *in vitro* neutralisation data, the inventors decided to examine the effects of 1C2 mAbs *in vivo*. The following describes the experiments that were performed to evaluate the efficacy of 1C2 mAbs for neutralising viruses in the lungs of mice, a model indicative of the usefulness of the antibody in other animals (including humans).

1.1 Methods

1.1.1 1C2 production

1.1.1.1 1C2 Hybridoma production

The hybridoma expressing 1C2 was produced as described by Morgan *et al.* [(1987) *Journal of Genetic Virology* 68, 2781-2788], using a protocol similar to that described previously by Routledge *et al.* [Routledge *et al.* (1985) *Journal of Medical Virology* 15, 305-320].

1.1.1.2 Hybridoma cell culture

Reagents:

RPMI 1640 containing 10% FCS

500ml RPMI medium with L-glutamine and sodium bicarbonate (Sigma, Cat. N^o. R8758), 50ml foetal calf serum (Sigma Cat. N^o. F4135), 5ml 200mM L-glutamine, 5ml P/S solution, mixed and stored at 4°C.

Procedure:

1C2 hybridoma cells, which produce murine IgG mAb specific for the G glycoprotein of the RSV (A strain specific) (see Section 1.1.1) were cultured in complete RPMI 1640 medium supplemented with 10% FCS, at 37°C, 5% CO₂, in either 25 cm² or 75 cm² or 225 cm² plastic tissue culture flasks, depending on the quantity of antibody required.

1.1.2 Neutralisation experiments

To evaluate the efficacy of 1C2 mAbs for neutralising viruses in the lungs of mice, BALB/c mice were injected with the 1C2 mAb and challenged with an intranasal inoculation of infectious RSV. Several days later, the quantity of infectious virus in the lungs of the mice was determined compared to the quantity in lungs of control mice.

1.1.2.1 Intranasal infection of mice with RSV

High titre infectious RSV A2 strain (infectious titre 2.72×10^8 ffu/ml¹), used for mouse infection experiments was provided by the Department of Microbiology and Immunology, University of Newcastle upon Tyne. The method used to produce the high titre virus is

described by Hayes *et al.* (Hayes, P.J., Scott, R. & Wheeler, J. (1994). *Journal Med Virol.* **42**, 323-329).

6-week old female BALB/c mice (maintained by the Comparative Biology Centre, University of Newcastle upon Tyne) were intranasally inoculated under fluorothane anaesthesia with 100 μ l of a suspension containing 1.36×10^8 ffu ml^{-1} of virus in HeLa cell maintenance medium. The mice were killed by cervical dislocation 4 days after inoculation, and the lungs removed to assess the extent of virus replication.

1.1.2.2 Intravenous injection of mice with monoclonal antibodies

In experiments designed to test the prophylactic protective effect of anti-RSV G monoclonal antibodies, mice were injected 24 hours prior to intranasal RSV infection with 200 μ l of sterile PBS containing 1 mg of purified mAb. 'No mAb' control mice received only PBS.

Groups of three mice were injected intravenously with single 1mg doses of either Protein A purified 1C2 (Example 4, section 4.1.5) or a heavy chain isotype matched (IgG2a) control anti-Adenovirus mAb clone 10/5.1.2 (produced by Novocastra laboratories, UK) in sterile PBS. 'No antibody control' mice were simply injected with 100 μ l of sterile PBS. Approximately 24 hours post antibody administration, the mice were inoculated intranasally with 1.36×10^7 ffu of RSV. Four days post inoculation, the mice were killed and the quantity of virus in the lungs was assessed.

1.1.2.3 Lung Homogenate preparation

Lungs removed from mice were placed in pre-weighed tubes containing 2 ml of cold RPMI medium (Gibco BRL) supplemented with 10% FCS. The tubes were re-weighed to determine lung weight, then lungs were homogenised in a sterile homogeniser.

After clarification by centrifugation for 5 min at 400 x g, 4°C, each homogenate was immediately assayed for infectious virus. The remaining undiluted homogenate was snap-frozen and stored at -70°C as back up.

1.1.2.4 Virus Titration (fluorescent focus assay)

200 μ l volumes of HeLa cell suspension containing 3×10^5 cells ml^{-1} in growth medium were seeded into the wells of a 96 flat well tissue culture plate (Costar) and incubated in 5 % CO₂, at 37°C overnight.

The following day, the cell monolayers were rinsed once with 200 μ l of warm PBS, and then inoculated with 25 μ l of clarified lung homogenate (either neat or diluted in 10-fold steps in maintenance medium). Each homogenate dilution was tested in duplicate wells. 'No virus' control wells were mock inoculated with maintenance medium. After inoculation, the plate was incubated in 5 % CO₂, at 37°C for 90 min. The inoculum was then removed and the monolayers rinsed again with PBS. 200 μ l of warm maintenance medium was added to each well and incubation was continued for 24 hours. The medium was then discarded and the cells were fixed with 200 μ l of cold 75 % acetone in PBS at 4°C for 10 min. After removal of the acetone and drying of the plate, 25 μ l of a 1/10 RSV mAb pool (hybridoma cell culture supernatant containing mAb 2G12₂ [anti-P], mAb 1C3 [anti-N], mAb 5H5 [anti-22K] and

mAb 5A6 [anti-FI) was added to each well. The plate was incubated at 37°C for 30 min in a moist box. The primary antibody was discarded and each well was washed twice with 200 µl of PBS, 5 min per wash. Next, 25 µl of a 1/50 dilution (in PBS) of sheep anti-mouse FITC conjugate (Novocastra) was added to each well and the plate incubated and washed as before. 200 µl of 0.0033 % naphthalene black was added to each well and the plate left for 10 min at room temperature. The wells were then washed as before with PBS, followed by a 1 min wash in 200 µl of deionised water, after which the plate was allowed to dry. The foci of infection were counted at 200x magnification using a fluorescence microscope, and the infectious titre of each virus sample was expressed in focus forming units per gram of lung tissue.

1.1.2.5 Controls

During the assay, two homogenisation controls were set up using mice that had been given either PBS or 1C2, but which had not received intranasal RSV. After dissection, the lungs from these mice were mixed with 1.36×10^7 ffu of infectious RSV and the put through the lung homogenisation and assay procedure. The objective of this exercise was to check that residual 1C2 in the blood released from the lungs was insufficient to neutralise RSV present during the homogenisation procedure.

1.2 Results

Figure 1 shows the geometric mean quantity of RSV recovered from the lungs of groups of 3 mice 4 days post intranasal challenge with virus. 24 hours prior to challenge, the mice were given a single intravenous dose of the indicated anti-RSV G or control antibody, or PBS. The error bars indicate the \pm 1 S.D. range of the means.

No significant difference was observed between the titres of virus recovered from the 'no antibody' (PBS only) control mice and the mice that received the anti-Adenovirus control antibody; the mean titres were 3.79×10^4 and 6.33×10^4 ffu g⁻¹ of lung respectively.

In contrast, no virus was recovered from the lungs of any of the three mice that had received mAb prior to RSV intranasal challenge. The result was significant at the 90% confidence interval.

The result of the homogenisation control indicated that infectious virus does not get neutralised in the lungs of mAb-inoculated mice during the lung homogenisation procedure. Consequently, the lack of virus in the mAb mice could only be due to the mAb having successfully blocked infection of the lungs when the intranasal virus challenge was given.

1.3 Discussion

The aim of this example was to investigate whether or not 1C2 was effective as a prophylactic agent against RSV infection. The quantity of infectious virus in the lungs of the mice was determined and compared to the quantity in the lungs of control mice by injecting BALB/c mice with the mAb, and then challenging them with an intranasal inoculation of infectious RSV.

Initial experiments suggested that 1C2 had poor *in vitro* neutralization activity (relative to mAbs specific for the RSV F protein) and its potential as a prophylactic or therapeutic reagent was not investigated at this time. However, in the course of this study, the inventors, to their surprise, have now discovered that the antibody has good efficacy *in vivo* for neutralising RSV virions.

Given this surprising discovery the inventors proceeded to characterise 1C2 in more detail with the aim of identifying key sequences and designing antibodies or derivatives thereof, which may be used according to the invention.

Genetic engineering procedures for antibody humanisation were employed to optimise the antibodies. First, a mouse-human chimaeric version of 1C2 using V_H and V_L regions of the heavy and light chains genes was produced (see Example 4). Future modification may be achieved by creating a reshaped version of mAb 1C2 consisting of the CDRs grafted onto a human IgG molecule. Prior to the production of such a chimaeric molecule, 1C2 was further characterised which involved the isolation of V_H and V_L regions of the heavy and light chains genes (see Example 2).

EXAMPLE 2

Given that the inventors realised how useful 1C2 may be (Example 1), they proceeded to further characterise 1C2. This Example provides an account of how isolation and subsequent sequencing of 1C2's heavy and light chain variable regions were achieved. This Example also describes how an scFv antibody carrying V_H and V_L from 1C2 was produced. Such an scFv antibody represents an antibody derivative, which may be used according to the invention.

2.1 Methods

General methodology is described below whereas specific procedures (primers, restriction enzymes, vectors, etc.) are described in section 2.2.

2.1.1 1C2 V_H and V_L Gene Sequence

2.1.1.1 *Preparation of total RNA from 1C2 hybridoma cells*

Total RNA was extracted from 10⁷ 1C2 hybridoma cells using an RNeasy kit (Qiagen; UK) following the manufacturer's protocol for isolation of total RNA from animal cells. 600µl volumes of lysis buffer and 70% ethanol were used. The RNA was eluted into a final volume of 30µl of DEPC-treated water and stored at -70°C. All the buffers were supplied in the kit.

2.1.1.2 *RT – PCR of immunoglobulin gene*

2.1.1.2.1 *cDNA synthesis: One step reverse transcription*

Synthesis of cDNA was carried out in a 20µl reaction mixture containing 5µl of total RNA. 2µl each of 10x RT buffer (50 mM tris-HCl pH 8.3, 50 mM KCl, 10mM DTT, 0.5 mM spermidine; Promega, UK), 25 mM MgCl₂ (Perkin-Elmer, USA), 10 mM dNTP (MBI Fermentas, UK) and 25 µM anti-sense primer (primer EJK193 and EJK194 for cDNA synthesis of 1C2 heavy and light chain variable regions, respectively), 5µl water, 0.5µl of RNasin ribonuclease inhibitor (40Uµl⁻¹; Promega, UK) and 1.5µl of AMV-RT (Promega, UK). The reaction tube was incubated at 42°C for 60 min, followed by heating at 95°C for 5 min and subsequent cooling down to 4°C in a Peltier thermal cycle model PTC-200 (MJ Research, USA). The cDNA was then immediately amplified by PCR (see 2.1.1.2.2) or stored at -20°C.

2.1.1.2.2 *Polymerase chain reaction (PCR)*

cDNA of interest was amplified via polymerase chain reaction on a programmable heating block (Peltier thermal cycle model PTC-200 (MJ Research, USA). The PCR primers used are described in table 1 and the results section 2.2.

Typically, 10µl of cDNA reaction product synthesized as described above was amplified in a reaction volume of 100 µl containing 10 µl of 10x PCR buffer (Promega, UK), 10 µl of 25 mM MgCl₂ (Promega, UK) 2 µl of 10 mM dNTP mixture (MBI Fermentas, UK), 5 µl of 25 µM of each of the appropriate two primers (sense and antisense primers), 56 µl of DEPC-treated water and 6 U of *Taq* DNA polymerase (Promega, UK).

Table 1: Oligonucleotides used for RT-PCR of 1C2 V_H & V_L genes.

Primer	Oligo sequence, 5'→3'	Sense	Use	Seq ID
EIK193	5' GTCTATCCACTGGCCCTG 3'	-ve	RT primer to synthesize mouse 1C2 H-chain cDNA	13
EIK194	5' ACTGGATGTGGGAAGATGG 3'	-ve	RT primer to synthesize mouse 1C2 kappa chain cDNA	14
CM195	5' CCGCTCGAGACGGTGACCGTGGTCCCTGGCCCCA 3'	-ve	PCR primers to isolate mAb 1C2 V _H gene for cloning 1C2 scFv gene	15
CM196	5' CGTGGCATGACACAGGTCGAGTTCGAGTCWGG 3'	+ve		16
CM197	5' CCGTTATGCGGCCGCGGAGATCTCCAGCTTGTGCC 3'	-ve		17
CM198	5' CGCTAAGTGCACAGGACATTCAGTACCCAGTCT 3'	+ve		18
CM215	5' CGCTAAGTGCACAGGATGTTTGTATGACCCARACT 3'	+ve		19
CM223	5' GCGGTATGTCACCTCCGATGTTTGTATGACCCAG 3'	+ve		20
CM224	5' CACCGAACGTCACGGAATATGTGAACC 3'	-ve		21
CM225	5' CCGCGCACGTCGCGCGCGGACCAAGCTGGAAA	+ve		22
CM226	5' GATCCGAATCTTAACACTCTCCCTGTTGAAGCT	-ve		23

2.1.1.3 *Agarose gel electrophoresis of DNA*

Reaction samples from RT-PCR were analysed on a 2% agarose gel aiming to isolate the bands comprising amplified V_H and the V_L regions of the heavy and the light chain gene, respectively.

2.1.1.3.1 *Reagents*

TBE buffer (10x)

108 g tris-(hydroxymethyl) methylamine, 55 g boric acid (Fisons), 40 ml of 0.5 M EDTA pH 8.0, dH₂O to 1 litre, dissolved autoclaved.

DNA loading buffer (6x)

0.05 % (w/v) bromophenol blue and 60% (w/v) glycerol, 5 mg of bromophenol blue (Electran), 6 g of glycerol, dH₂O to 10ml, dissolved and stored at 4°C in 1.0 ml aliquots.

DNA size standard markers

*Eco*R I and *Hind* III-digested Lambda DNA molecular weight marker solution (NBL, UK) was adjusted to a concentration of 35.0 ng μ l⁻¹ in agarose gel sample buffer. *Hae* III-digested ϕ X174 DNA molecular weight marker solution (Kramel Biotech, UK) was prepared at a concentration of 100 ng μ l⁻¹ in agarose gel sample buffer. Both solutions were stored at -20°C. The sizes of *Eco*R I and *Hind* III-digested λ DNA fragments are 21.226, 5.148, 4.877, 4.268, 3.530, 2.027, 1.904, 1.584, 1.375, 0.947, 0.831, and 0.564 kbp. The sizes of *Hae* III-digested ϕ X174 DNA are 1.353, 1.078, 0.872, 0.603, 0.310, 0.281, 0.271, 0.234, 0.194, 0.118, and 0.072 kbp.

2.1.1.3.2 *Procedure*

DNA fragments were separated by electrophoresis in 0.8-2.0 % w/v agarose gels (depending on the size of DNA fragment to be isolated) prepared in TBE buffer, using a horizontal gel electrophoresis apparatus.

2.1.1.3.2.a *Preparation of agarose gels*

The appropriate amount of agarose (GibcoBRL) was melted in 50 ml of 1x TBE buffer in a boiling water bath. The gel was cooled to 60°C, ethidium bromide was added to a final concentration of 1.0 μ gm μ l⁻¹, then the gel was poured into a gel tray containing a suitable well template, and allowed to set.

2.1.1.3.2.b *Preparation gel electrophoresis*

15 μ l of 6x sample buffer was added to the preparative restriction enzyme digested DNA (to give a final volume of 90 μ l). The mixture was then electrophoresed on an appropriate agarose gel at 60 volts for 1-2 hours.

2.1.1.3.2.c *Analytical gel electrophoresis*

2 μ l of 6x sample buffer was added to the analytical restriction enzyme digested DNA to give a final volume of 12 μ l). The mixture was then electrophoresed on an appropriate agarose gel at 60 volts for 1 hour.

The resulting DNA fragments were monitored and photographed under ultraviolet (UV) light. 12 µl of *EcoR* I and *Hind* III-digested λ DNA solution of 5 µl of *Hae* III-digested φX174 DNA solution, prepared in 1x sample buffer, was loaded onto preparative or analytical gels as molecular weight markers.

2.1.1.4 Purification of DNA fragments from agarose gels

The DNA fragments separated in preparative gels were visualized using long wavelength UV light. The required band was sliced from the gel using a sterile scalpel blade. The DNA was extracted from the gel slice by either using a Qiagen gel extraction kit, or by electroelution.

For electroelution, the gel slice was placed in a dialysis tube containing 0.5x TBE buffer, submerged in a horizontal gel electrophoresis tank in 0.5x TBE buffer and then electrophoresed at 110 volts for 30 minutes. The eluate (approximately 400 µl) was collected from the dialysis bag and transferred to a microcentrifuge tube. Subsequently, the DNA solution was extracted once with 400 µl of phenol, once with 400 µl of a 1:1 mixture of phenol and chloroform, and once with 400 µl of chloroform. At each extraction step the DNA solution was vortexed for 2 min and centrifuged for 2 min, then the upper aqueous layer carefully transferred to a fresh microfuge tube for the next stage. After the last chloroform extraction, the DNA was precipitated by adding 20 µl of 5 M NaCl and 1 ml of -20°C ethanol. After incubation at -70°C for 30 min, the DNA was pelleted by centrifugation for 20 min.

The supernatant was removed and the pellet was rinsed in 1 ml of 70% ethanol at room temperature. The tube was respun for 5 min and the supernatant was removed. After a short spin for 30 seconds to remove the residual supernatant the DNA pellet was dried in a 45°C heat block. The resulting DNA was finally resuspended in 20µl of sterile deionised water.

2.1.1.5 Restriction enzyme digestions

Amplified (see section 2.1.1.2.2) and purified cDNA (see section 2.1.1.4) was subjected to restriction enzyme digestion. Restriction enzyme digestion was carried out as part of the preparation for ligation of amplified genetic material into vectors and for subsequent analysis confirming the presence of inserted genes within plasmid vectors.

2.1.1.5.1 Preparative restriction enzyme digestions

Approximately 5-10 µg of DNA was digested with 30 units of the desired enzyme (or enzymes) in the appropriate reaction buffer (obtained from the enzyme manufacturer) in a final volume of 75µl. The reaction mixture was then incubated at 37°C for 2 hours (or 16 hours if recommended by the manufacturer).

In the case of DNA being cut with more than one enzyme with incompatible reaction requirements (i.e. different temperature of buffer composition), the enzymes were used sequentially. In between each digestion, DNA was precipitated by adding 2.5 volumes of ethanol and 1/20th volume of 5M NaCl. After incubation for 30 min at -70°C, DNA was pelleted by centrifugation. The pellet was rinsed once with 70 % ethanol (in water), dried, resuspended in 75 µl of the next restriction enzyme buffer (at 1x strength), and then the next restriction enzyme digestion was carried out.

2.1.1.5.2 Analytical restriction enzyme digestions

Approximately 0.5-1 µg of DNA was digested with 5 units of the appropriate enzyme (or enzymes) in the appropriate buffer, in a final volume of 10µl. The reaction mixture was then incubated at 37°C for 2 hours.

2.1.1.6 DNA ligation reactions

Fragments generated as described in sections 2.1.1.2 – 2.1.1.5 were inserted into vectors as explained below.

For a ligation reaction, approximately 0.1 µg of vector DNA was mixed with a 2-fold or 5-fold excess (approximately) of insert DNA and 1.2 units of T4 DNA ligase (GibcoBRL). The reaction was carried out in 1x ligation buffer in a final volume of 12 µl at 16°C for 16 hours. Simultaneously, a 'vector only' negative control reaction was set up in an identical fashion, except that the insert DNA was omitted and replaced with sterile water. The ligated DNA was stored at -20°C if required.

2.1.1.7 Transformations of *E.coli* with plasmid vectors

Plasmids as specified later on carrying the gene fragments of the heavy and light chain variable regions were used to transform competent *E.coli* TG1 suppressor strain cells in order to allow the production of phage antibody particles at a later stage.

Competent *E.coli* TG1 suppressor strain cells (K12, Δ (*lac-pro*), Sup E, thi, hsd D5/F', traD 36, pro AB, lacIq, lac Z-ΔM15) were prepared using the Hanahan standard transformation protocol.

After a ligation reaction, the 12 µl of ligation mixture was topped up to 100 µl by adding 88 µl of sterile water. 210 µl aliquots of competent bacteria were added to 3x 15 ml polypropylene tubes (Falcon 2059) and placed on ice.

10 µl of each of three dilutions (1/10, 1/100 and 1/500) of the ligation mixture were separately added to each tube. The bacteria were then incubated on ice for 30-40 min. Subsequently, the bacteria were heat-shocked by placing the tubes in a 42°C water bath for 90 seconds. Afterwards, the tubes were cooled on ice for 2 min.

After heat shock and cooling, 0.8 ml of SOB medium (Hanahan, 1985) was added and the tubes were incubated at 37°C for 30 min to allow the bacteria to start expressing drug resistance. Finally, 100µl volumes of the bacteria were plated out onto TYE agar plates (containing 100 µgm⁻¹ ampicillin, and with [for TG1 or HB2151 transformed with pHEN2 vector] or without 1% glucose) using a glass spreader, and incubated overnight at 37°C. Individual colonies of interest were then picked from the plate and re-streaked on a fresh medium plate and incubated as before. A well-isolated individual colony was then picked from each re-streak plate using a sterile pipette tip. The colonies were inoculated into tubes containing 10 ml of 2x TY medium with 100 µgm⁻¹ ampicillin. These were incubated at 37°C overnight, to provide sufficient bacteria for plasmid DNA isolation by the miniprep extraction method (see section 2.1.1.8)

2.1.1.8 Mini-preparation of plasmid DNA

The cells from an overnight bacterial culture (see section 2.1.1.7) were collected by centrifugation at 4,000 x g for 15 minutes. Plasmid DNA was extracted from the pellet using a Wizard Plus SV Miniprep DNA purification system (Promega, UK) according to the manufacturer's instructions. The plasmid DNA was eluted into a final volume of 50 µl of nuclease-free water.

2.1.1.9 Maxi-preparation of plasmid DNA

Where required in large quantities (e.g. for transfection experiments), plasmid DNA was isolated from the bacteria collected from 500 ml of overnight culture (2x TY medium containing 100 µgml⁻¹ ampicillin) using the alkaline lysis, lithium chloride / polyethylene glycol (PEG) precipitation and phenol / chloroform extraction procedures described by Sambrook *et al.* (Sambrook J., Fritsch E., Maniatis T. (1989), *Molecular Cloning*, 2nd ed. pp 1.33-1.41. New York: Cold Spring Harbour Laboratory Press). After the phenol / chloroform extraction step, the DNA solution was precipitated with ethanol and ammonium acetate.

The DNA was collected by centrifugation, rinsed with 70 % ethanol and dried, then dissolved in 200 µl of TE buffer. The DNA concentration was determined by spectrophotometry.

2.1.1.10 DNA sequencing

DNA was prepared for sequencing at a concentration of 0.2 µgµl⁻¹ in DEPC-treated water. Approximately 10 µl of DNA was required per sequencing reaction.

Sequencing was performed by the University of Newcastle upon Tyne Molecular Biology Facility Unit, using a PE applied biosystem, ABI PrismTM 377 DNA sequencer. The primers used to sequence the various genes involved in these examples are described in tables 2 and 3.

They were prepared at a concentration of 1.6 µM. Each sequencing reaction required 10 µl thereof. The M13 forward and reverse primers were supplied by the University of Newcastle upon Tyne Molecular Biology Facility Unit.

Table 2: Sequencing primers used to sequence scFv antibody genes.

Primer	Primer sequence (5'→3')	Sense	Description (Primer annealing to)	Seq ID
LMB3	5' CAG GAA ACA GCT ATG AC 3'	+	DNA upstream of pelB leader in pHEN2 vector	24
Fdseq1	5' GAA TTT TCT GTA TGA GG 3'	-	gIII coat protein DNA in pHEN2 vector	25
FOR_LinkSeq	5' GCC ACC TCC GCC TGA ACC 3'	-	linker sequence in pHEN2 vector	26
pHEN-Seq	5' CTA TGC GGC CCC ATT CA 3'	-	DNA downstream of c-myc-tag in pHEN2 vector	27

Table 3: Sequencing primers used to sequence mutant human IgG CD3 mAb heavy chain gene (derived from L15P61) and 1C2 chimaeric heavy and light chain genes.

Primer	Primer sequence (5'→3')	Sense	Description (Primer annealing to)	Seq ID
SN 123	5' GGA GAT AGT GAA TCG GCC 3'	-	Framework Region 3 of humanised YTH12.5 CD3 mAb V _H gene	28
SN 124	5' AGG ACA GCC GGG AAG GTG 3'	-	C _H 1 domain of human γ 1 gene	29
SN 125	5' TCT TCG TGG CTC ACG TCC 3'	-	C _H 2 domain of human γ 1 gene	30
SN 126	5' TGA CCA GGC AGG TCA GGC 3'	-	C _H 3 domain of human γ 1 gene	31
SN 130	5' TCA GCC TGA CCT GCC TGG 3'	+	C _H 3 domain of human γ 1 gene	32
CM 229	5' GAT TTC ACA CTC AAG ATC 3'	+	FR3 region of mouse 1C2 V _K II gene	33
CM 230	5' CAA CTG CTC ATC AGA TGG 3'	-	C domain of human K gene	34
CM 231	5' TAT AGG CTG TGC TGG AGG 3'	-	FR3 region of mouse 1C2 V _H	35

2.1.1.11 Phage antibody (scFv) rescue from 96 well microtray cultures of TGI/phagemid bacteria

Following the ligation of the 1C2 V_L (Kappa) gene into vector p525 (see Fig. 2) and transformation of *E. coli* TG1, ampicillin resistant clones were selected, cultured as described in section 2.1.1.7 and subsequently subjected to the microtray 'phage rescue' protocol to induce the production of phage antibody particles.

A selection of individual TGI/phagemid bacterial colonies recovered after antigen selection of phagemid particles and subsequent infection of *E. coli* TG1 (see section 2.1.1.7), were individually inoculated into 100 μ l of 2x TY-Amp-Glu medium in the wells of a 96 well flat-bottomed tissue culture plate (Corning, USA). The same colonies were simultaneously inoculated onto a single TYE-Amp-Glu plate in a recorded grid pattern to produce a replica plate. The culture plate was shaken overnight, and then 2 μ l of each culture was transferred to a new plate containing 200 μ l of the same medium per well. After shaking for 1 hour, 10⁹ p.f.u of M13K07 helper phage in 25 μ l of 2x TY-Amp-Glu were added to each well. The

helper phage M13 K07, used to rescue (package) phagemid vectors from *E. coli* TG1 as M13 phage particles, was obtained from Amersham Pharmacia Biotech (Cat. N° 27-1524-01).

The plate was incubated for 30 min and then shaken for a further 1 hour. The infected bacteria were pelleted by centrifugation at $1,800 \times g$ for 10 min and the supernatant was discarded. The pellet in each well was resuspended into 200 μ l of 2x TY containing 100 μ gml⁻¹ ampicillin and 50 μ gml⁻¹ kanamycin. After overnight incubation at 30°C in the orbital shaker, the bacteria were again pelleted by centrifugation and 50-100 μ l of the culture supernatant was assayed for the presence of phage antibodies by ELISA as described in section 2.1.1.12 (table 2.4(A)). The bacterial cultures were incubated at 37°C unless otherwise stated.

2.1.1.12 ELISA

To assist the further characterisation of 1C2 mAbs, the identities of its heavy and light chains were determined by ELISA.

2.1.1.12.1 Reagents

Substrate buffer

5.106 g citric acid, 18.426 g Na₂HPO₄·12H₂O, dH₂O to 1 litre, dissolved, adjusted to pH 5.0, autoclaved.

OPD Substrate solution (62.5mgml⁻¹O)

1.25 g OPD (Sigma), 20 ml of substrate buffer, dissolved and dispensed into 0.4 ml aliquots, stored at -20°C.

Complete OPD substrate (freshly prepared)

0.4 ml of OPD substrate solution, 25 ml of substrate buffer and 10 μ l of H₂O₂.

PBST (0.05%) (Washing buffer)

PBS pH 7.4, containing 0.05% v/v Tween 20 (Sigma)

3M H₂SO₄

83.4 ml concentrated H₂SO₄, added into 416.6 ml of dH₂O.

2.1.1.12.2 Procedure

Depending on type or properties of the antigens and antibodies that were being assayed, many variations of a basic ELISA protocol were used throughout this project. The details of the different assays, i.e. the dilutions of the reagents that were used, the composition of blocking buffers and the length of incubation periods, are given in table 5. The basic protocol is described below.

The wells of Nunc maxisorb flat bottomed 96 well ELISA plate were coated overnight at room temperature with the appropriate capture antigen or antibody diluted in PBS. After rinsing, wells were blocked (where a blocking step was included) for 2 hours at 37°C with 200 μ l of the appropriate blocking buffer. The plates were rinsed again, and then incubated sequentially with remaining assay reagents, with rinsing in between each step to remove non-bound material. Unless otherwise stated, the volume of each reagent applied per well was 50 μ l, incubation periods were carried out at 37°C for 1 hour each, and each rinse procedure consisted of 4 individual washings of the plates. PBST 0.05 % was used as the rinse buffer, and PBST (0.05 %) containing 1% BSA was usually used as the reagent diluent unless

otherwise stated. After application and rinsing off of the final peroxidase-conjugated reagent, the ELISA was developed by adding 100 μ l of complete OPD substrate solution to each well. After 30 min at room temperature, the reaction was stopped by the addition of 100 μ l of 3 M H_2SO_4 . Subsequently, the absorbance of each well's contents was measured at 492 nm using a Microplate Reader (Revelation version 4.02, Dynex Technologies).

Table 4: Immunoreagents used for ELISA experiments

IMMUNOREAGENTS	SOURCE
i) Polyclonal antibody <ul style="list-style-type: none"> Goat anti-human IgG, Fc-specific 	Sigma (I2136)
ii) Monoclonal antibodies <ul style="list-style-type: none"> Mouse anti-RSV-G mAb (1C2) Mouse anti-RSV-F mAb (1A12) Mouse anti-RSV-F mAb (5A6) Mouse anti-c-myc mAb (culture supernatant from 9E10 hybridoma cells) <ul style="list-style-type: none"> A pool of mouse anti-RSV mAbs [Culture supernatants from anti-RSV-N (1C3), anti-RSV-P (2G12), anti-RSV-F (5A6) and anti-RSV-22K (5H5)] Mouse anti-Adenovirus mAb (clone 10/5.1.2) Mouse anti-polyhistidine mAb (Clone His-1) 	Dr. G.L. Toms Department of Microbiology and Immunology European Collection of Cell Cultures Code No 85102202 Novocastra Laboratories UK (NCL-RSV3) Novocastra Laboratories UK (NCL-Adeno) Sigma (H-1029)
iii) Conjugated antibodies <ul style="list-style-type: none"> Goat anti-mouse IgG (γ-chain specific) peroxidase Extravidin peroxidase Mouse anti-human kappa L-chain-biotinylated Goat anti-mouse Ig peroxidase Sheep anti-mouse fluorescein-thiocyanate Sheep anti-human Ig-biotinylated Anti-M13 peroxidase 	Sigma (A3673) Sigma (E2886) Pharmingen (34172D) Novocastra Laboratories, UK (NCL-GAMP) Novocastra Laboratories, UK (NCL-SAM-FITC) Amersham Pharmacia biotech (RPN1003) Pharmacia biotech (27-9411-01)

Table 5: ELISA

Step	Assay	(A) Antigen-specific detection of anti-G and anti-NIP phage antibodies	(B) Antigen-specific detection of anti-G and anti-NIP soluble scFvs
Step 1. Antigen coated on the Plate		BSA-NIP or BSA or BSA-G or 10xconc. or RSV-infected or uninfected (100 µgml ⁻¹) (50 µgml ⁻¹) HB2151/p417 HB2151/pHEN2 HeLa cell antigen (pHENG3) culture supernatant (20 µgml ⁻¹) (20 µgml ⁻¹) (diluted 1/2 in PBS)	Antigens as listed in section A
Step 2. Plate blocking step		<div style="display: flex; align-items: center;"> <div style="border: 1px solid black; padding: 2px; margin-right: 10px;"> PBS + 3% w/v BSA (for anti-NIP or IC2 phage antibodies) </div> <div> 10 mM Tris-HCl pH 8.5 + 1% betaine + 2% w/v dried skimmed milk + 10% w/v BSA for Griffini1 library phage antibodies </div> </div>	PBS + 3% w/v BSA
Step 3. Second layer	RT 90 min	<div style="display: flex; align-items: center;"> <div style="border-left: 1px solid black; border-right: 1px solid black; padding: 0 10px;"> Phage antibody (from <i>E. coli</i> TG1 culture) Either unpurified culture supernatant or PEG-concentrated. (diluent was the blocking buffer indicated above in step 2) </div> <div style="margin-left: 10px;"> or neat mAb IC2 hybridoma culture supernatant (for positive antigen control well) </div> </div>	Soluble scFv (from <i>E. coli</i> HB2151 culture supernatant) Either unpurified or purified (RT - 60 min)
Step 4. Third layer	RT 90 min	<div style="display: flex; align-items: center;"> <div style="border-left: 1px solid black; border-right: 1px solid black; padding: 0 10px;"> 1/5,000 anti-M13 peroxidase conjugate (diluted in appropriate blocking buffer) </div> <div style="margin-left: 10px;"> or 1/2,000 goat anti-mouse IgG (γ-chain) peroxidase conjugate in PBS + 1% w/v BSA (for positive antigen control well) </div> </div>	1/100 mouse anti-c-myc or 1/3,000 mouse anti-polyhistidine (RT - 60 min)
Step 5. Fourth layer		None	1/2,000 goat anti-mouse IgG (γ-chain) peroxidase conjugate (RT - 60 min)

Table 5: ELISA (continued)

Step	Assay	(C) Detection of 1C2 chimaeric mAbs (via human H & L chain constant regions)	(D) Detection of anti-G binding activity in 1C2 chimaeric mAbs
Step 1. Antigen or antibody coated on the plate		1/2,000 goat anti-human IgG Fc antibody	BSA-G or BSA or RSV-infected or Uninfected HeLa cell antigen (20 μgml^{-1}) (100 μgml^{-1}) (20 μgml^{-1})
Step 2. Plate blocking step		None	PBST (0.05%) + 10 % FCS
Step 3. Second layer		<div> <div>37°C 90 min</div> <div>Transfected NS0 cell culture supernatant (neat or titrated in 4-fold steps)</div> <div>or</div> <div>Protein-A or purified chimaeric antibody</div> <div>or</div> <div>Campath 1H (1 μgml^{-1})</div> </div> <div> <div>Titrated in 4-fold steps</div> </div>	<div> <div>Protein-A purified 1C2 chimaeric mAb</div> <div>or</div> <div>Protein-A purified mouse 1C2 mAb</div> </div> <div> <div>Titrated in 4-fold steps</div> </div>
Step 4. Third layer		1/4,000 sheep anti-human IgG or 1/4,000 mouse anti-human kappa biotinylated conjugate	<div> <div>1/4,000 sheep anti-human IgG biotinylated conjugate</div> <div>or</div> <div>1/2,000 goat anti-mouse IgG (γ-chain) peroxidase conjugate</div> </div>
Step 5. Fourth layer		1/1,000 extravidin peroxidase conjugate	<div> <div>1/1,000 extravidin peroxidase conjugate</div> <div>or</div> <div>None</div> </div>

2.1.1.13 Western blotting

2.1.1.13.1 Reagents

Immunoreagents (see section 2.1.1.12)

Protein blotting buffer pH 8.3

12.12 g tris (hydroxymethyl) methylamine, 58 g glycine, 800 ml methanol, dH₂O to 4 litres.

PBST (0.1%) (Washing buffer)

4x PBS pH 7.4 containing 0.1% v/v Tween 20.

2.1.1.13.2 Reagents used for Western blotting for protein sequencing

10x CAPS stock buffer; 100 mM CAPS pH 11.0

22.13 g 3-[cyclohexylamino]-1-propanesulphonic acid (CPAS; Sigma), dissolved in 900 ml deionised H₂O, adjusted to pH 11.0 with approximately 20 ml of 2 N NaOH, dH₂O to 1 litre, stored at 4°C.

Protein blotting buffer

100 ml 10 x CAPS buffer, dH₂O to 1 litre.

Protein blotting buffer-0.002% w/v DTT

100 ml of 1 x CAPS buffer, 2 mg DTT (Sigma).

Protein stain

2 g Coomassie blue R-250 (Sigma), 100 ml acetic acid, 500 ml methanol, dH₂O to 1 litre gently heated and stirred until the Coomassie blue powder had completely dissolved.

Destain solution

100 ml acetic acid, 500 ml methanol, dH₂O to 1 litre.

2.1.1.13.3 Procedure

After SDS PAGE, each gel was equilibrated in 100 ml of protein blotting buffer for 20 min at room temperature with gentle shaking. A piece of Schleicher & Schuell BA85 nitrocellulose paper (for blots described for immunostaining) or PVDF membrane (for blots described for protein sequencing) with dimensions 1 cm larger than the gel was soaked in transfer buffer for 30 min prior to use.

Electrophoretic transfer of the proteins from the gel into nitrocellulose paper or nylon membrane was carried out as described by Towbin *et al.* (Towbin, H., Staehelin, T. and Gordon, J. (1979). *Proc Natl Acad Sci USA*, 76, 4350-4354), using a BioRad Western blotting tank, for 3 h at 100 V. Once transfer was complete the orientation of samples on the membrane with respect to the gel was marked with a pencil.

Nitrocellulose blots (for immunostaining) were briefly washed in PBST (0.1%), then, incubated overnight at 37°C in 100 ml of blocking buffer containing 0.1% NaN₃ unless otherwise stated. After overnight blocking, they were washed three times in 100 ml of washing buffer for 5 min each and then re-blocked in 50 ml of fresh blocking buffer for 1 hour at room temperature.

Nylon membrane blots (for protein sequencing) were rinsed with deionised water containing DTT (0.002%, w/v), and then stained for 30 min with Coomassie blue R-250. After destaining, the rinsing step was repeated and the blots were soaked in the rinsing solution for 10 min. Finally, the blots were dried, and the appropriate bands cut out for sequence analysis carried out at the University of Newcastle upon Tyne facility for Molecular biology. All steps were carried out at room temperature.

2.1.1.14 Staining of Western blots

After re-blocking, the blots were treated with a variety of different combinations of immunoreagents, depending on the nature of the blotted protein that was being stained. The different reagents, the reagent diluents, and the dilutions at which the reagents were used are summarized in table 6.

'No primary antibody' control blots were treated with antibody diluent. Unless indicated otherwise in table 6, a mixture of a 1/10, 000 dilution of goat anti-mouse peroxidase conjugate (Novocastra) and a 1/10,000 dilution of biotinylated streptavidin peroxidase complex (Sigma, E2886), was used as the secondary detector antibody, and staining steps were performed at room temperature for 1 hour with rinsing in between each step to remove excess reagents. The appropriate blocking buffer without dried skimmed milk was usually used to dilute primary and secondary antibodies.

To avoid bacterial contamination, sodium azide was added to primary antibodies (never to peroxidase conjugate) at the final concentration of 0.1%. PBST (0.1%) was used as the rinse buffer and each rinse procedure consisted of three individual 100 ml washes of 5 min each, followed by an additional wash for 15 min.

After staining and rinsing off of the peroxidase-conjugated reagent, ELC (RPN 2109) or ECL plus (RPN 2132) Western blotting reagent (Amersham Pharmacia Biotech) were used to develop stained blots, following the manufacturer's protocol.

The proteins on the membrane were visualized by exposing Hyperfilm ECL high performance autoradiography film (Amersham Pharmacia Biotech) to the blot membranes for periods of between 30 sec and 5 min, based on signal appearance. The film was developed with film developer (Kodak) and film fixer (Kodak).

Table 6: Western blotting

Steps	Assay	(A) Detection of 1C2 scFv antibody in culture supernatant or cell extract of IPTG-induced <i>E. coli</i> HB2151	(B) Detection of 1C2 soluble scFv antibody purified from a periplasmic extract of HB2151/p525
Step 1. Antibody blotted onto nitrocellulose membrane		<p>10x conc. or 10x conc. or 10x conc. or Unconc. or HB2151/ HB2151/ HB2151/ HB2151/ p528 p525 p525 p525 pHEN-NIP pHEN-NIP (frameshift-corrected 1C2 scFv)</p> <p>↓ culture supernatant</p> <p>↓ culture supernatant or cell extract</p>	<p>Unpurified insoluble / soluble bacterial periplasmic extract</p> <p>Fractions recovered from Talon (IMAC) column</p>
Step 2. Membrane blocking step		<p>↓</p> <p>PBST (0.1 %) + 5 % w/v dried skimmed milk (4°C - overnight)</p> <p>↓</p> <p>PBST (0.1 %) + 5 % w/v dried skimmed milk + 1 % w/v BSA + 10 % v/v FCS</p>	PBST (0.1 %) + 5 % w/v dried skimmed milk + 1 % w/v BSA + 10 % v/v FCS
Step 3. Primary antibody staining (37°C-1 hour)		1/100 mouse anti-c-myc mAb in blocking buffer (without dried skimmed milk)	1/100 mouse anti-c-myc mAb in blocking buffer (without dried skimmed milk)
Step 4. Secondary antibody staining (37°C-1 hour)		1/10,000 goat anti-mouse Ig peroxidase conjugate (Novocastra) + 1/10,000 extravidin peroxidase conjugate	1/10,000 goat anti-mouse Ig peroxidase conjugate (Novocastra) + 1/10,000 extravidin peroxidase conjugate

Table 6: Western blotting (continued)

Steps	Assay	(C) Detection of mouse 1C2 mAb and mouse / human chimaeric 1C2 mAb
Step 1. Antibody blotted onto nitrocellulose membrane		Protein-A purified mouse 1C2 mAb or Protein-A purified mouse / human chimaeric 1C2 mAb (0.8 μgml^{-1})
Step 2. Membrane blocking step		PBST (0.1 %) + 5 % w/v dried skimmed milk + 1% w/v BSA + 10 % v/v FCS
Step 3. Primary antibody staining		None
Step 4. Secondary antibody staining		1/10,000 goat anti-mouse IgG peroxidase conjugate (Novocastra) or 1/5,000 sheep anti-human IgG biotinylated conjugate + 1/10,000 extravidin peroxidase conjugate
Step 5. Tertiary antibody staining		None \downarrow 1/10,000 extravidin peroxidase conjugate \downarrow

2.2 Results

2.2.1 **Determining the isotype of the 1C2 mAb heavy and light chain.**

The isotype of 1C2 heavy and light chains were determined by ELISA (section 2.1.1.12). The absorbance values produced when the assay was developed with pNPP (p-nitrophenyl phosphate) substrate are shown in table 7. The result indicated that 1C2 is an IgG2a_c antibody.

This information was used to design 2 oligonucleotide primers, EJK 193 and EJK 194 (see table 1) which are complementary to mouse gamma 2a (γ2a) and mouse kappa (κ) constant region mRNA respectively.

Table 7: Isotyping of the 1C2 mAb heavy and light chains.

<u>Test Specificity</u>	<u>Absorbance at 405 nm</u>
λ	0.181
κ	2.269
γ1	0.172
γ2a	>3.0
γ2b	0.137
γ3	0.211
μ	0.217
α	0.160

Using an isotype kit obtained from Pharmingen (Cat. N^o 04017K, Becton Dickinson, UK) following the manufacturer's protocol, the culture supernatant from the 1C2 mAb hybridoma cell line was spot tested in wells coated separately with 8 different mouse immunoglobulin isotype-specific rat monoclonal capture antibodies, then detected with rat anti-mouse immunoglobulin alkaline phosphatase conjugate.

2.2.2 **The strategy employed to sequence 1C2**

The sequences of the variable regions of both, the heavy and the light chain of 1C2 were determined. Initially, the 1C2 V_H and V_L variable regions of the heavy and light chain genes were isolated using RT-PCR. The total RNA extracted from 1C2 hybridoma cells was used as the template for reverse transcription and subsequently amplified variable regions of the heavy and light chain genes were inserted into a phagemid expression vector pHEN2 (see <http://www.mrc-cpe.cam.ac.uk/g1p.php?page=1808> for sequence) thereby creating a 1C2 scFv construct, which was expressed as a soluble scFv or phage antibody in *E. coli*.

2.2.2.1 *Isolation of 1C2 total RNA*

Total RNA was isolated from 1C2 producing hybridoma cells as described in section 2.1.1.1. A sample of 1 µg of the total RNA was analysed by electrophoresis. The RNA was of good quality, there being no obvious sign of degradation by RNAses.

2.2.2.2 *Isolation and Sequencing of the 1C2 variable region of the heavy chain gene*

The next step required the synthesis of 1C2 V_H gene cDNA from the RNA by using primer EJK193 (see table 1) in a reverse transcription (RT) reaction (see section 2.1.1.2). The cDNA was then amplified by PCR using primers CM195 (see table 1) and CM196 (see table 1). Their 5' ends were designed to incorporate restriction enzyme sites required to facilitate subsequent ligation of the PCR product into the pHEN2 vector. The RT-PCR was performed as described in section 2.1.1.2 and reaction samples were subsequently analysed on a 2% agarose gel (see section 2.1.1.4).

This analytical step revealed two unique bands at approximately 383 bp and at approximately 220 bp. The major band, which was the size expected for a V_H gene, was isolated by gel electrophoresis and gel extraction, digested with *Nco* and *Xho* I, then re-purified by gel electrophoresis and extraction. The resulting DNA fragment was ligated into *Nco* I – *Xho* I digested pHEN2. The ligation products were then used to transform *E. coli* TG1. Plasmid DNAs were isolated from 16 of the resulting ampicillin resistant bacterial colonies, and digested with *Nco* I and *Xho* I. All 16 clones carried a 370 bp insert. The inserts of five of the clones were sequenced using primers LMB3 (see table 1) and fdSeq1 (see table 1) (see section 2.1.1.10) and then cross-compared. All five clones were found to carry the same V_H gene. No variability was seen in the sequences of the five clones, except in the 5' oligo primer region, which was presumably caused by the redundancy built into CM196. The clone chosen for further experiments was named p516.

A comparison of the theoretical amino acid sequence of the 1C2 V_H gene with the Kabat database indicated that it was a mouse subgroup II A heavy chain variable region gene. Clone p516 was selected for further work, because in this clone the N-terminal amino acid sequence of the V_H gene (the region encoded by the 5' oligo primer) was identical to the corresponding region of mouse V_H II A genes.

2.2.2.3 *Sequencing of the 1C2 variable region of the light chain gene*

2.2.2.3.1 *Isolation of the 1C2 variable region of the light chain gene*

cDNA of the 1C2 V_L gene was prepared from 1C2 hybridoma total RNA using primer EJK194 in the reverse transcription reaction, then amplified using primers CM197 and CM215 in the subsequent PCR (see section 2.1.1.2 and table 1). As with the V_H gene, PCR primers CM195 and CM 196, primers CM197 and CM215 were modified at their 5' ends to facilitate ligation of the PCR product into pHEN2. The same PCR conditions as those used for isolating the 1C2 V_H gene were used (section 2.1.1.2).

In the RT stage of the RT-PCR, both the EJK194 primer annealing step and the RT incubation step (1 step RT) were performed at 42°C followed by PCR amplification. Subsequent gel electrophoresis of the PCR product revealed a single band of approximately 352 bp.

2.2.2.3.2 Ligation of the 1C2 V_L (Kappa) gene into p525 and Transformation of *E. coli* TGI

2.2.2.3.2.a Assembly of p525

Initial attempts to ligate cDNA of the 1C2 V_L gene region into *Apa*I - *Not* I cut p516 (i.e. pHEN2 carrying the 1C2 V_H gene see section 2.2.2.2) produced clone p528. However, subsequent transformation of *E. coli* HB2151 (non-suppressor strain (K12, *ara*, D (*lac-pro*), *thi* /F⁺ *proA*⁺B⁺, *lacI*^q ZDM15) (Carter, P., Bedouelle, H. and Winter, G. (1985). *Nucleic Acids Res.* 13, 4431-4443) indicated poor production of scFv antibodies comprising the variable regions of both, the heavy and the light chain genes whereas the controls vector pHEN2-NIP produced large amounts of scFv. pHEN2-NIP is an anti-NIP phagemid based on pHEN2, but carrying a human V_H and V_L gene combination specific for the hapten 3-nitro-4-hydroxy-5-iodophenylacetic acid (NIP). It was supplied by the MRC as a positive control for use in expression experiments.

When the DNA sequences of p528 and pHEN-NIP were rechecked from the *pelB* leader sequence to the amber stop codon at the *c-myc* tag / gene 3 protein gene junction, it became surprisingly apparent that a change of one amino acid (alanine, A, to aspartate, D) had been introduced into the *pelB* leader peptide sequence, at the leader peptide / scFv junction which was later found to be caused by a mistake in the design of primer CM196.

To investigate whether it was this amino acid substitution that induced poor 1C2 scFv production levels, a gene swap experiment was carried out. Therein, the *Pst* I - *Not* I 1C2 scFv gene insert of p528 was cloned into *Pst* I - *Not* I cut pHEN-NIP vector and, vice versa, the *Pst* I - *Not* I anti-NIP scFv gene from pHEN-NIP was cloned into *Pst* I-*Not* I - cut p528 vector. When isolating the *Pst* I - *Not* I 1C2 scFv gene, p528 had to be partially digested with *Pst* I because the 1C2 scFv gene possesses an internal *Pst* I site

This gene swapping exercise produced two new phagemids. The first (p525) consisted of pHEN2 carrying the 1C2 scFv gene with an alanine (A) residue as the last amino acid in the *pel B* leader. The second (p526) consisted of pHEN2 carrying the anti-NIP scFv gene with an aspartate (D) residue as the last amino acid in the *pel B* leader.

Subsequently, these two plasmids were individually introduced into *E. coli* HB2151 cells. Samples of bacterial cell extracts prepared by sonication (6 ml cell suspension exposed to two 30 second 10 micron bursts in a Soniprep 150 sonicator at an interval of 2 min), and centrifugation at 13,000 rpm for 5 min to remove the cell debris, along with samples of concentrated culture supernatants were analysed by Western blotting followed by immunostaining with anti-*c-myc* detector antibody (see sections 2.1.1.13 and 2.1.1.14, respectively).

The results of this experiment indicated that changing the aspartate residue back to alanine improved the production of the 1C2 scFv whilst substituting alanine for aspartate in pHEN2-NIP caused a drop in anti-NIP scFv production.

As this confirmed the importance of the alanine residue at the last position in the Pel B leader sequence, plasmid p525 was used as vector accepting the V_L region of the light 1C2 chain in further experiments.

2.2.2.3.2.b Ligation of 1C2 V_L region of the light chain gene into p525

The band detected during gel electrophoresis in section 2.1.1.4 was extracted from the gel, purified, digested with *Apa*I and *Not*I, re-purified and ligated into *Apa*I – *Not*I-cut p525 (the pHEN2 clone containing the 1C2 variable region of the heavy chain gene with the alanine residue in the pel B leader sequence)(see section 2.1.1.6). After transformation into *E. coli* TG1, 20 ampicillin resistant clones were selected and cultured in the wells of a 96 well tissue culture plate (see section 2.1.1.7). The cultures were subjected to the microtray 'phage rescue' protocol to induce the production of phage antibody particles as described in section 2.1.1.11. The neat culture supernatants from the tissue culture plate (containing the phage antibodies) were then spot tested for antigen binding specificity by ELISA, on BSA-G and BSA control antigen. The results thereof provided positive antigen binding results for fifteen of the clones selected. The two producing the highest specific absorbance values (clones p529 and p530) were re-streaked onto ampicillin plates to ensure that they were pure clones.

It will be appreciated that the construct carried by plasmid p530 comprises an scFv construct, which upon expression can be used to neutralise RSV (see Fig. 3).

2.2.2.3.3 Sequencing of p530

Plasmid DNA was extracted from large-scale cultures of these two clones by the maxiprep procedure (see section 2.1.1.9) and the DNA was subsequently sequenced using primers LMB3 and fdSeq1 to confirm the presence of, and to determine the nucleotide sequence of the 1C2 V_K gene (see section 2.1.1.10).

The sequence, alongside its theoretical amino acid translation, is shown in Fig.3 and is identified as Sequence ID N^o44 (Sequence ID N^o45 corresponds to the amino acid sequence derived therefrom).

As it can be appreciated from Fig. 3, the V_H and V_L regions are essential to the invention and their sequences are given in Fig. 4 and Fig. 5, respectively.

EXAMPLE 3

This example illustrates that the scFv phage antibody, which is the functional derivative of 1C2 according to the present invention, is able to bind to the RSV G glycoprotein. Furthermore, it describes how the binding specificity of the scFv 1C2 antibody encompassing the variable regions of the heavy and the light chain was determined. Overall, this example shows that the scFv phage antibody would be suitable for use *in vivo*.

3.1 Methods

Previously, in section 2.2.2.3.2.b, following scFv expression, neat culture supernatants from a tissue culture plate containing the phage antibodies were spot tested for antigen binding specificity by ELISA on BSA-G and BSA control antigen. The clones producing the highest specific absorbance values of positive antigen binding were then selected for subsequent sequence analysis of the phage antibody.

In this Example, a competitive ELISA was used to demonstrate the binding specificity of the scFv antibody.

3.1.1 Competitive ELISA

To prove that the binding activity observed in ELISA when using the 1C2 phage antibody (p530) was specific, a competitive ELISA illustrating the binding of 1C2 scFv phage antibodies was carried out as described in 2.1.1.12 using the anti-Adenovirus mAb 10/5.1.2, and mAb 1C2 as the competitors.

75 μ l aliquots of PBS containing 10^{13} t. u. of 1C2 scFv phage antibody were mixed with equal volumes of PBS containing a range of concentrations of the two competitor mAbs, and a final concentration of 3% BSA. The mixtures were then incubated in ELISA plate wells (coated with BSA-G antigen and blocked) for 90 min at room temperature. After discarding the mixtures and rinsing the plate, the relative quality of 1C2 phage antibody bound to each well was assayed using anti-M13 peroxidase conjugate and ELISA substrate.

3.2 Results

The result (figure 6) showed that the binding of the 1C2 scFv phage antibody to the BSA-G antigen was only blocked by the 1C2 mAb (i.e. conventional 1C2 IgG2a). This confirmed that the binding specificity of the 1C2 scFv phage antibody was the same as that of the original 1C2 hybridoma mAb.

3.3 Discussion

A competitive ELISA using the anti-Adenovirus mAb 10/5.1.2, and mAb 1C2 as the competitors was carried out to prove that the binding activity observed in ELISA when using the 1C2 scFv phage antibody (p530) was specific.

The results showed that the binding specificity of the scFv 1C2 phage antibody was the same as that of the original 1C2 hybridoma mAb and thereby confirmed that the variable regions of both, the heavy and the light chain gene have successfully been isolated.

This also showed that the scFv phage antibody according to the present invention has a strong potential for the treating RSV infection.

EXAMPLE 4

This example describes the production of two mouse-human IgG1 chimaeric antibodies using the V_H and V_L genes of 1C2. Both antibodies possess identical human kappa light chain and human $\gamma 1$ heavy chain constant regions, except that one of the antibodies carries an Asn²⁹⁷ → Ala mutation in its heavy chain C_H2 domain. This amino acid substitution removes the antibody's heavy chain N-linked carbohydrate attachment site, rendering the antibody 'aglycosyl'.

The purpose of producing these two 1C2 chimaeric antibodies was to reduce antibody antigenicity in humans and thereby produce preferred antibodies according to the invention.

4.1 Methods

See methods of Example 1 for:

PCR	1.1.3.2.2
Restriction enzyme digestions	1.1.3.3
Agarose gel electrophoresis of DNA	1.1.3.4
Purification of DNA fragments from agarose gels	1.1.3.5
DNA ligation reactions	1.1.3.6
Transformations of <i>E. coli</i>	1.1.3.7
ELISA	1.1.3.12
DNA sequencing	1.1.3.10

4.1.1 *Plaque purification of M13 bacteriophage stock*

This technique was used in the construction of the human $\gamma 1$ and aglycosyl $\gamma 1$ chimaeric 1C2 heavy chain genes during experiments confirming the successful execution of sequence changes of the L15P61 vector by site-directed mutagenesis (section 4.1.3). It was applied to purify a clone (M607) following site directed mutagenesis which enabled its subsequent growth as a culture facilitating the purification and sequence analysis of double stranded RF DNA in the *E. coli* cells of the culture (section 4.1.2).

10 μ l of culture medium containing the appropriate infectious phage particles was serially diluted in 10-fold steps from 10^{-1} – 10^{-9} , in 90 μ l aliquots of 2xTY medium. 250 μ l of overnight TG1 culture and 4 ml of soft TY agar (at 43°C) were added and vortexed with each dilution, then overlaid onto TYE plates. The plates were incubated inverted overnight to allow plaques to develop.

4.1.2 *Intermediate scale (Midi-) preparation of M13 RF bacteriophage DNA*

Plaques developed overnight on the plates described in section 4.1.1 were isolated and used to infect of *E. coli* TG1 culture followed by purification of and sequence analysis of double stranded RF DNA in the *E. coli* cells of the culture.

4.1.2.1 *Reagents*

Solution I, Solution II, and Solution III:

Same as described in section 2.1.1.9 for the large-scale preparation of plasmid DNA (Sambrook *et al.* (1989), *Molecular Cloning*, 2nd ed. pp 1.33-1.41. New York: Cold Spring Harbour Laboratory Press).

Low Salt Buffer: 0.2 M NaCl, 20 mM Tris-HCl pH 7.3-7.5, 1.0 mM EDTA, 20 ml 5.0 M NaCl, 10 ml 1.0 M Tris-HCl pH 7.4, 1 ml 0.5 M EDTA, dH₂O to 500 ml, dissolved and autoclaved.

High salt buffer: 1.0 M NaCl, 20 mM Tris-HCl pH 7.3-7.4. 1.0 mM EDTA, 10 ml 5.0 M NaCl, 1 ml 1M Tris-HCl pH 7.4, 0.1 ml 0.5 M EDTA, dH₂O to 50 ml, dissolved and autoclaved.

4.1.2.2 *Procedure*

One well-spaced plaque from a plaque purification procedure was picked and added to 50 ml of 2xTY broth containing 2 ml of an overnight TG1 culture, then shaken at 200 rpm for 7 h at 37°C. The infected bacteria were pelleted by centrifugation at 4,500 rpm in a MSE Mistral bench top centrifuge for 15 min and stored at -20°C, the supernatant was collected and stored either at 4°C or -20°C for further use if required.

To extract the RF DNA, the bacterial pellet was thawed, resuspended in 2 ml of plasmid preparation solution I, and vortexed. 4 ml of plasmid preparation solution II was then added, and mixed by inverting the tube. After 5 min incubation on ice, 3 ml of plasmid preparation solution III was subsequently added and mixed as above, and incubation continued in ice for a further 5 min. The contents were then transferred to a 14 ml Falcon tube (code 2059) and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was transferred to a new 50 ml Falcon tube and vortexed with an equal amount of 1:1 phenol:chloroform/isoamylalcohol for 2 min. After centrifugation at 4,500 rpm for 20 min at room temperature, the aqueous phase was transferred to a 30 ml Corex® tube and the nucleic acid precipitated by addition of a 0.7 volume of isopropanol. The nucleic acid was recovered by centrifugation at 10,000 rpm for 20 min at room temperature. The supernatant was discarded and the pellet gently rinsed with 5 ml of 70 % ethanol. The remaining of 70 % ethanol was removed after a quick spin, the pellet was dried at 37°C, then resuspended in 0.5 ml of TE buffer pH 8.0 containing 20 µgml⁻¹ RNase. The DNA was purified using an Elutip column (Schleicher & Schuell) according to the manufacture's instructions, then resuspended in TE buffer at a final concentration of 5 mg of nucleic acid per ml.

4.1.3 *Site-directed mutagenesis*

In vitro site-directed mutagenesis to make coding changes or to insert and delete restriction enzyme sites was carried out using the Quickchange™ Site-Directed Mutagenesis Kit (Stratagene, USA). This technique is a PCR-based procedure, which requires a pair of complementary oligos for each mutation (or cluster of mutations) that is to be introduced. Only one pair of complementary oligos can be used per reaction. Consequently, in instances where several widely separated mutations had to be introduced into a gene, multiple rounds of mutagenesis were necessary. Mutagenic oligonucleotides containing the desired mutations were designed and the mutagenesis reactions were performed according to the manufacture's protocol. Details of the mutagenic oligos are described in table 8. The product of the mutagenesis reactions were used to transform either *Epicurian coli*® XL1-Blue supercompetent cells (provided in the kit) or laboratory-made *E. coli* competent cells.

Table 8: Mutagenic oligonucleotides used to correct the nucleotide deletion in the 1C2 (type VI) V_L gene, and to construct the 1C2 chimaeric H and L chain genes.

Gene mutated	Mutagenic oligo	Oligo sequence, 5'→3'	Sense	Mutation introduced	Seq ID
1C2 V _L gene	CM202	5' GTAATCCACCCACATTCGGTGGTGG 3'	+	Insertion of an "A" nucleotide into the 1C2 V _L gene to create the Thr ⁹⁷ codon	36
	CM203	5' CCAGCACCAGAAATGGGTGGATTAC 3' (complementary to CM202)	-		37
Human IgG CD3 heavy chain gene	ER131	5' CAGGTGTCCACTCCACAGTGCCAA CTGCAGGAGTCTGGGGGCG 3' <i>Pst</i> I	+	Introduction of a <i>Pst</i> I site and a Glu ¹ -Gln substitution mutation	38
	CM216	5' CGCCCCCAGACTCCTGCAGTTGGACCT GGGAGTGGACACCTG 3' (complementary to ER131)	-		39
Human IgG CD3 heavy chain gene	CM217	5' CTCCTCAGCAGCGTCGTGACCGTGCCCTCC 3' <i>Bst</i> E II deleted	+	Removal of the unwanted C _H 1 domain <i>Bst</i> E II-site	40
	CM218	5' GGAGGCACGGTCACGACGCTGCTGAGGGAG 3' (complementary to CM217)	-		41
L15P61 plasmid	CM221	5' CAGCTACAGGTGTGCACTCCGAGGTCC 3' <i>Apa</i> L I	+	Introduction of an <i>Apa</i> L I-site into the 3' end of Ig leader peptide sequence	42
	CM222	5' GGACCTCGGAGTGCACACCTGTAGCTG 3' (complementary to CM221)	-		43

4.1.4 Transfection of NS0 cells with immunoglobulin gene expression vectors

For the production of the 1C2 chimaeric antibodies, the mouse myeloma cell line NS0 was chosen.

4.1.4.1 Media and Reagents

Iscove's Modified Dulbecco's Medium (IMDM) stock

35.32 g Iscove's modified Dulbecco's medium (GibcoBRL, Cat N° 42200-030), 6.05 g NaHCO₃ (BDH), dH₂O to 2 litres, dissolved, sterilized by filtration through a 0.2 µm membrane, and stored at -20°C.

IMDM containing 20% FCS (for growing NS0 cell line)

158 ml sterile IMDM stock, 2 ml Penicillin/Streptomycin solution (GibcoBRL, Cat. N°. 15140-122), 40 ml foetal calf serum (GibcoBRL), mixed and stored at 4°C.

Dialyzed Fetal calf serum

100 ml foetal calf serum (Sigma; F4135), dialyzed against 4 x 4 litres of sterile-PBS pH 7.4 over 48 hours, at 4°C, filter-sterilized through a 0.2 µm membrane and stored in 20 ml aliquots at -20°C.

50x GS supplement (200 ml); all ingredients supplied by Sigma

100 ml 100 x non-essential amino acid (NEAA) (Sigma; M7145), 702 mg L-asparagine monohydrate, 603 mg L-glumatic acid monosodium salt, 70 mg adenosine, 70 mg guanosine, 70 mg cytidine, 70 mg uridine, 24 mg thymidine, 2 ml 5N NaOH, mixed and incubated in 37°C water bath until dissolved, made up to a final volume of 200 ml with sterile-PBS, filter-sterilized through a 0.2 µm membrane and stored in 20 ml aliquots at -20°C. The pH should be about 7.0-8.0.

Non-selective medium (DMEM containing 20% FCS)

154 ml glutamine-free DMEM (JRH Bioscience, Cat N° 51435-79P), 2 ml 100x NEAA (Sigma), 2 ml 200mM L-glutamine, 2 ml P/S solution, 40 ml FCS (Sigma; F4135; not dialyzed), mixed and stored at 4°C.

Selective medium (G-DMEM containing 20% dialysed FCS)

152 ml glutamine-free DMEM (JRH Bioscience, Cat N° 51435-79P), 2 ml P/S solution, 6 ml 50x GS-supplement, 40 ml dialyzed FCS, mixed and stored at 4°C.

4.1.4.2 Procedure

NS0 mouse myeloma cells (obtained from Dr. E. Routledge, Department of Microbiology and Immunology, University of Newcastle upon Tyne) were maintained in exponential growth phase as semi-adherent monolayers in 75 cm² plastic tissue culture flasks, using IMDM supplemented with 20% FCS and 1% P/S.

Prior to transfection, 100 µg of the appropriate expression vector plasmid DNA was linearized by digestion with *Sal* I for 16 hours at 37°C (2 units of enzyme per µg of DNA), in a 650 µl reaction volume.

The DNA was then extracted with phenol / chloroform, then with 1 ml of water-saturated ether. After removal of the ether phase, the DNA solution was incubated at 45°C for 10 min, and then precipitated by the addition of a 1/10 volume of 3 M sodium acetate and 3 volumes of -20°C ethanol.

After mixing and overnight incubation at -70°C, the DNA precipitate was recovered by centrifugation (13,000 rpm for 15 min at room temperature), rinsed twice with 200 µl volumes of -20°C 70% ethanol, dried then resuspended in 100 µl of sterile PBS. The DNA concentration was determined and adjusted to 0.5 µgµl⁻¹ by the addition of extra sterile PBS.

On the day of transfection, monolayers of adherent NS0 cells were gently rinsed twice with 10 ml of sterile PBS, then detached from the plastic into 5 ml of fresh PBS by tapping the flask against the palm of the hand. The cells were then pelleted by centrifugation (1,200 rpm for 5 min) in a 50 ml plastic centrifuge tube, washed twice in 50 ml volumes of ice-cold PBS, then finally resuspended in fresh cold PBS to a concentration of 1.5×10^7 cells in 900 µl.

The 900 µl of the cells and 100 µl of linearized DNA solution at 0.5 µgµl⁻¹ (50 µg) were mixed together in a chilled 0.4 cm electroporation cuvette (BioRad) and held on ice for 5 min. The cell / DNA mixture was then given two consecutive 0.1 millisecond pulses of 1,500 volts, 3 µF with a 15 second interval in between the two pulses. After electroporation, the cells were held on ice for a further 12 min then added to 40 ml of warm non-selective NS0 cell growth medium. The suspension was then distributed into the wells of eight 96 well flat-bottomed tissue culture plates (50 µl per well) and incubated at 37°C, 5% CO₂. On day 2 post-transfection, 110 µl of warm NS0 cell selective medium was added to each well, and incubation was continued. On day 4 or 5 post-transfection, 100 µl of medium was removed from each well and replaced with fresh selective medium. The plates were returned to 37°C until substantial cell death and discrete surviving colonies were observed. Once the medium in growth-positive wells had begun to turn orange-yellow in colour, 50 µl volumes of culture supernatant were removed for ELISA testing to detect the presence of 1C2 chimaeric antibody using goat anti-human IgG Fc (Sigma, I2136) as capture antibody.

If selected for culture expansion on the basis of antibody production levels, NS0 cell cultures from the 96 well plates were individually transferred into the wells of 24 well plates, then 6 well plates, then 25 cm² tissue culture flasks, over a period of approximately 2 weeks. Cell samples from expanded cultures were frozen in liquid nitrogen, in FCS containing 8.3% DMSO.

The two transfected NS0 cell cultures selected for large-scale chimaeric 1C2 mAb production were further expanded into 75 cm² tissue culture flasks and from there into roller bottles. Selective medium was used throughout the culture expansion process until the roller bottled had been seeded, after which normal IMDM containing 10% FCS and 1% P/S was used.

4.1.5 Purification of immunoglobulin by Protein A Sepharose affinity chromatography

4.1.5.1 Reagents

250 mM Tris-HCl pH 8.0

30.25 g tris (hydroxymethyl) methylamine, 900 ml dH₂O, dissolved and adjusted to pH 8.0 with concentrated HCl, dH₂O to 1 litre, autoclaved and stored at 4°C.

1 M Tris-HCl pH 8.5 containing 0.02% NaN₃

8.8 g tris (hydroxymethyl) aminomethane hydrochloride (BDH), 5.3 g tris (hydroxymethyl) methylamine, 0.1 ml 20 % NaN₃, dH₂O to 100 ml, dissolved, adjusted to pH 8.5 with 5 M NaOH, autoclaved and stored at 4°C.

100 mM Glycine

1.9 g glycine, 230 ml dH₂O, dissolved, adjusted to pH 2.8 with concentrated HCl, dH₂O to 250 ml, filter-sterilized through a 0.2 µm membrane, stored at 4°C.

2 M Urea containing 0.04% NaN₃

30.03 g urea (Sigma), 0.5 ml 20 % NaN₃, dH₂O to 250 ml, dissolved and filter-sterilized through a 0.2 µm membrane, stored at 4°C.

1M LiCl

10.6 g LiCl (Sigma), dH₂O to 250 ml, dissolved and filter sterilised through a 0.2 µm membrane and stored at 4°C.

4.1.5.2 Procedure

Column preparation: 10 ml of Protein A Sepharose 4B Fast flow (Sigma; P9424) was resuspended in 100 ml of PBS pH 7.4, gently swirled for 2 min, and left at room temperature for 30 min to allow the beads to be settle. The supernatant was removed and the equilibration step repeated with 100 ml of fresh PBS. After discarding the supernatant, the beads were resuspended in 20 ml of fresh PBS, and degassed under vacuum for 1 hour, then packed into a Pharmacia XK16/20 column.

Sample preparation: To avoid bacterial contamination sodium azide solution was added to monoclonal antibody-containing tissue culture supernatants to a final concentration of 0.1% w/v. The pH was then adjusted to 7.2-7.5 by adding 250 mM Tris-HCl pH 8.0.

Column chromatography: Between 5 and 12 litres of tissue culture supernatant (containing the mAb to be purified) was first passed through a 0.2 µm membrane filter capsule (Pual Gelman), then run through the packed Protein A Sepharose column at a flow rate of approximately 2 litres per day. The column was washed by running through 400 ml of PBS pH 7.4, then bound the immunoglobulin (antibody) was eluted using 0.1 M Glycine pH 2.8. 5 ml fractions of eluate were collected into tubes containing 1 ml of 1 M Tris-HCl pH 8.5, followed by gentle mixing to neutralize the pH of the eluted protein.

After use, the column was cleaned by sequentially running through 200 ml of PBS pH 7.4, 100 ml of 2 M urea, 200 ml of PBS, 100 ml of 1 M LiCl, and 200 ml of PBS containing 0.05% sodium azide. The column was stored at 4°C in between runs. The monoclonal antibody purified from the column was dialysed against (2 x 4 litres) PBS pH 7.4, and the protein concentration was then determined by measuring the absorbance at 280 nm. The antibody concentration was calculated using the formula below:

$$\text{Antibody concentration (mgml}^{-1}\text{)} = \text{OD}_{280\text{nm}} \times \text{dilution factor} / 1.35$$

4.1.6 *Polyacrylamide Gel Electrophoresis (PAGE)*

4.1.6.1 *Reagents*

2 x normal strength resolving gel buffer, pH 8.8

90.6 g tris (hydroxymethyl) methylamine, 2.0 g SDS, dH₂O to 1 litre, dissolved and adjusted to pH 8.8 using concentrated HCl.

2 x normal strength stacking gel buffer, pH 6.8

30.24 g tris (hydroxymethyl) methylamine, 2.0 g SDS, dH₂O to 1 litre, dissolved and adjusted to pH 6.8 using concentrated HCl.

20 mg ml⁻¹ ammonium persulphate solution

0.2 g ammonium persulphate, dH₂O to 10 ml, mixed and stored at 4°C for a maximum of one week.

40% Acrylamide – bisacrylamide (Ratio 19 : 1) stock solution

40% Acrylamide – bisacrylamide stock solution was obtained from BDH (Cat N° 443725R).

4x Reservoir buffer

24.2 g tris (hydroxymethyl) methylamine, 8.0 g sodium dodecyl sulphate (SDS), 115.2 g of glycine, dH₂O to 2 litres, mixed and diluted 1:4 in dH₂O to produce normal strength buffer.

0.1% w/v bromophenol blue solution

0.1 g bromophenol blue powder (BDH), dH₂O to 100 ml.

2x Reducing sample buffer

4 g SDS, 50 ml of 2 x normal strength stacking gel buffer, 20 ml of glycerol, 2 ml 0.1% w/v bromophenol blue solution, 5 ml of 2-mercaptoethanol, dH₂O to 100 ml.

Protein molecular weight markers

For SDS-polyacrylamide gels to be stained with Coomassie blue, a molecular weight standard mixture containing 25 µgml⁻¹ of the proteins listed in table 9 was prepared in 1x reducing SDS-PAGE sample buffer. 40 µl per well of the molecular weight markers was loaded onto the gel. For gels to be used in Western blotting, a biotinylated molecular weight standard mixture (Sigma SDS-6B) containing 0.1 µgml⁻¹ of the proteins listed in table 10 was prepared in 1x reducing SDS-PAGE sample buffer.

40 μ l per well of the molecular weight markers diluted 1/10 in 1x reducing sample buffer was applied to the gel. The molecular weight standard mixtures were usually dispensed in 100 μ l aliquots and stored at -20°C. The mixtures were heated in a boiling water bath for 2 min before application to the gel.

Table 9: Sizes of protein molecular weight markers used on Coomassie blue-stained SDS-PAGE gels.

Proteins	Approx. Molecular Weight (Daltons)
Myosin, rabbit muscle	205, 000
β -galactosidase, <i>E. coli</i>	116, 000
Phosphorylase b, rabbit muscle	97, 400 (Sigma, SDS-6H)
Albumin, bovine	66, 000
Albumin, egg	45, 000
Carbonic anhydrase, bovine erythrocytes	29, 000
Trypsin inhibitor	20, 100 (Sigma, T9767)
α -lactalbumin	14, 200 (Sigma, L6385)

Table 10: Sizes of protein molecular weight markers (SDS-6B, Sigma) used for Western blotting

Biotinylated proteins	Approx. Molecular Weight (Daltons)
Phosphorylase b, rabbit muscle	97, 400
Catalase, bovine liver	58, 100
Alcohol dehydrogenase, horse liver	39, 800
Carbonic anhydrase, bovine erythrocytes	29, 000
Trypsin inhibitor, soybean	20, 100
Lysozyme, chicken egg white	14, 300

4.1.6.2 Procedure

The sodium dodecyl sulphate (SDS) discontinues buffer system described by Laemmli (Laemmli (1970). *Nature*, 227, 680-685) was used in PAGE. 8%, 10% or 12.5% polyacrylamide resolving gels were prepared in a Protean II Electrophoresis Cell (BioRad Laboratories). Briefly, the appropriate resolving gel mixture (see table 11) with the exception of the TEMED was prepared in a side arm flask and deaerated under vacuum for approximately 5 min. The TEMED was then added and the mixture poured into the assembled electrophoresis cell and overlayered with a water-saturated butanol. When the gel had set, the butanol was discarded and the gel surface was rinsed with distilled water. The resolving gel was then overlayered with a 20 well 4% w/v polyacrylamide stacking gel.

Protein samples (in 1x SDS-PAGE reducing sample buffer) and SDS-PAGE protein MW markers were heated for 2 min in a boiling water bath and cooled to room temperature. Sample volumes of in between 20 and 100 μ l (depending on the gel run) were loaded into the wells of the gel, and then carefully overlayered with normal strength reservoir buffer. The reservoirs of the electrophoresis cell were then filled with reservoir buffer and electrophoresis was carried out using an LKB (Bromma) 2297 Macrodrive 5 constant power pack (LKB Produkter AB). The gel received a constant current of 45 mA for approximately 3 hours until the dye front was 1 to 0.5 cm from the bottom of the resolving gel. The electrophoresis cell was dismantled and the stacking gel was discarded. The resolving gel was fixed by immersion in Coomassie Blue stain (see below), or stained using silver staining kit (Pharmacia Biotech, Cat N° 17-1150-01), or equilibrated in protein transfer buffer for Western blot analysis.

Table 11: Volume of stock solutions in standard gel mixtures

Stock solutions	50 ml of Resolving gel			40 ml of Stacking gel
	8%	10%	12.5%	
Acrylamide-bisacrylamide solution (ml)	10	2.5	15.6	4
2x *NS resolving gel buffer (ml)	25	25	25	-
2x NS Stacking gel buffer (ml)	-	-	-	20
Distilled water (ml)	14	11.5	8.4	15
Ammonium persulphate solution (20 mgml ⁻¹) (ml)	1	1	1	1
Deaerate	yes	yes	yes	yes
NNN'N' tetramethylethylenediamine	15	15	15	15

* NS = normal strength

4.1.7 Coomassie blue staining of Polyacrylamide gels

4.1.7.1 Reagents

Protein stain

0.5 g Coomassie brilliant blue (Sigma), 100 ml glacial acetic acid, 250 ml isopropyl alcohol. dH₂O to 1 litre, gently heated and stirred until the Coomassie blue powder had completely dissolved.

Destain solution (45% methanol v/v)

450 ml methanol, 100 ml acetic acid, dH₂O to 1 litre.

4.1.7.2 Procedure

After electrophoresis, the resolving gel was immediately immersed in a 200 ml bath of Coomassie blue stain and incubated stationary overnight at room temperature. The next day the gel was washed two or three times in 200 ml volumes of destain solution for 3 hours per wash. When destaining was complete, the gel was stored in a bath of 10% acetic acid, or it was dried. All destaining incubations were performed with gentle shaking at room temperature.

4.2 Results

4.2.1 Production of two mouse-human IgG1 chimaeric antibodies

Production of the two chimaeric antibodies required the assembly of three different chimaeric immunoglobulin genes.

- (a) The first, necessary for both antibodies, was a gene encoding the 1C2 chimaeric light chain, consisting of the 1C2 V_L II variable region linked to the human C_L (Inv 3) constant region gene (Hieter *et al.* (1980) *Cell* **22**, 197-207).
- (b) The second, necessary for the production of 'normal' glycosylated 1C2 chimaeric antibody, was a gene encoding the 1C2 chimaeric heavy chain, consisting of the 1C2 V_H variable region linked to the human $\gamma 1$ (Glm, 1, 17) constant region gene (Takahashi *et al.*, (1982) *Cell* **29**, 671-679).
- (c) The third gene, necessary for the production of the aglycosyl 1C2 chimaeric antibody, was identical to the above chimaeric heavy chain gene except that a substitution mutation (AAC \rightarrow GCC, Asn \rightarrow Ala) had to be introduced at codon 297.

4.2.1.1 Construction of the human $\gamma 1$ and aglycosyl $\gamma 1$ chimaeric 1C2 heavy chain genes.

The cloning strategies devised for assembling the two chimaeric 1C2 heavy chain genes utilised a pre-existing M13 vector, L15p61. This vector contains a 1.3kbp cDNA encoding the $\gamma 1$ heavy chain gene of the humanised version of the anti CD3 mAb, YTH12.5 (Routledge, *et al.* (1991) *Eur. J. Immunol.* **21**, 2717-2725). The heavy chain gene in L15P61 carries a Ser⁴⁴⁴ \rightarrow Cys mutation in its C_H3 domain (Shopes, B. (1992). *J Immunol.* **148**, 2918-2922), and had been used to produce CD3 antibodies that could be dimerised via disulphide bond formation.

For the purpose of producing the 1C2 chimaeric heavy chain genes, the CD3 antibody V_H region gene was excised from L15P61, and replaced with the 1C2 V_H gene from the pHEN2-derived vector p530 (described in section 1.2.3). This switch produced the 1C2 V_H region with a 'Kozak' sequence for optimum translation efficiency in mammalian cells (Kozak, M. (1987). *Mol Biol.* **196**, 947-950). It also provided an immunoglobulin heavy chain leader signal sequence, necessary for protein secretion

from mammalian cells (the pel B leader sequence in p530, used to direct soluble scFv antibody secretion in *E. coli*, is not suitable for this purpose). The switch also linked the 1C2 V_H gene to the human γ 1 constant region carried by L15P61.

The 1C2 V_H gene in p530 is flanked at its 5' end by a unique *Pst* I site and at its 3' end by a unique *Bst*E II site. These sites can be used to isolate the V_H gene from p530. However, the same is not true of the CD3 V_H gene carried by L15P61. Although the 3' end *Bst*E II site is present, the 5' *Pst* I site is absent.

In addition, the 3' *Bst*E II is not unique: there is a second *Bst*E II site in the C_H1 domain exon of the human γ 1 gene. Consequently before the CD3 V_H gene in L15P61 could be swapped for the 1C2 V_H gene, the L15P61 vector had to be modified by site-directed mutagenesis. An initial mutagenesis reaction, using mutagenic oligos ER131 and CM216, was carried out to introduce a *Pst* I site into the 5' end of the CD3 V_H gene (at codon 3 and 4, i.e. the same position as the *Pst* I site in the 1C2 V_H gene). This mutagenesis reaction was also used to introduce a Glu¹ → Gln substitution mutation into the CD3 V_H gene, so that its 5' sequence immediately upstream of the *Pst* I site became identical to that of the corresponding region of the 1C2 V_H. The M13 clone (M605) isolated from this first round of mutagenesis was then subjected to a second mutagenesis reaction using oligonucleotides CM217 and CM218. This reaction was performed to delete the CD3 heavy chain C_H1 domain *Bst*E II site, by introducing a 'silent' coding change. Restriction enzyme analysis of 10 clones isolated from the second mutagenesis reaction indicated that 5 had successfully incorporated the *Bst*E II site deletion mutation, and still retained the *Pst* I site insertion mutation from the first reaction. One of these clones (M607) was plaque-purified and grown as a 50 ml culture. The double stranded RF DNA in the *E. coli* cells of the culture was purified using an Elutip, then sequenced using the primers M13 forward, SN123, SN124, SN125 and SN126 (see Example 2, table 2.7). Analysis of the sequence confirmed that all of the desired sequence changes, and no unwanted changes, had been incorporated during the mutagenesis reactions.

The next stage of the gene assembly process was to switch the CD3 V_H gene in M607 for the 1C2 V_H gene from p530. Consequently, the 1C2 V_H gene was isolated from p530 by digestion with *Pst* I and *Bst*E II, purified by gel electrophoresis, then ligated into *Pst* I – *Bst*E II digested M607 vector DNA. The ligation product was used to transform *E. coli* TG1. RF DNA was extracted from 5 of the resulting plaques and digested with *Pst* I – *Bst*E II enzymes. All of them showed an insert band of the expected size (344bp). 'Elutip' purified RF DNA from one of the clones (M608) was sequenced using the primers listed earlier and also the M13 reverse primer, to confirm that it really did carry the 1C2 V_H gene (NB. The 1C2 and CD3 V_H genes cannot be distinguished on the basis of size on an agarose gel).

Although the chimaeric heavy chain gene in M608 was essentially complete, it still carried the unwanted Ser⁴⁴⁴ → Cys mutation in its C_H3 domain. One possible way of removing this mutation was to use site-directed mutagenesis to convert the Cys⁴⁴⁴ codon back to Ser. A further round of mutagenesis could then be used to introduce the Asp²⁸⁷ → Ala change in C_H2 domain to produce the aglycosyl γ 1 heavy chain gene. However, because of the availability of two other vectors in the laboratory, L51P71 and M501b, it was decided that the easiest way to achieve these changes was to carry out a constant region domain swap procedure. L51P71 is a derivative of the

plasmid pEE6 which carries a non-mutant version of the humanised CD3 γ 1 heavy chain cDNA; i.e. it lacks the Ser⁴⁴⁴ → Cys mutation (NB: L51P71 could not be used instead of L15P61 for the initial V_H gene exchange procedure described earlier, because pEE6 contains a *Sac* II site which would interfere with the subsequent C region strategy).

M50lb is a derivative of M13mp18, which carries an aglycosyl version of the CD3 γ 1 heavy chain cDNA; i.e. it lacks the Ser⁴⁴⁴ → Cys mutation, but also possesses the Asn²⁹⁷ → Ala substitution. (NB; M50lb was not used for the initial V_H gene exchange procedure because it lacks a Kozak sequence.)

To repair the Ser⁴⁴⁴ → Cys mutation in M608 and also to introduce the Asn²⁹⁷ → Ala aglycosyl mutation, the *Sac* II – *Eco*R I segment of M608 DNA encompassing the γ 1 C_{H2} and C_{H3} domains was removed and replaced with the corresponding segment of DNA from either L51P71 or M50lb. These two final ligations resulted in the production of clones M609 and M610, which carried the completely assembled chimaeric 1C2 γ 1 and aglycosyl 1C2 γ 1 heavy chain genes respectively. The structure of both genes was confirmed by partial sequencing using the primers SN126 and M13 forward (The sequences of the two chimaeric heavy chain genes are illustrated in Figure 3 within Example 2).

4.2.1.2 Construction of the 1C2 human kappa chimaeric light chain gene.

The 1C2 chimaeric light chain gene was assembled using components from three different vectors; L15P61, p530 and p347. L15P61, as previously described, is an M13 based vector, which contains a 1.4 kbp γ 1 heavy chain cDNA from the humanised CD3 mAb YTH12.5. L15P61 was used as a vector backbone in which the light chain was assembled. It also provided the Kozak and immunoglobulin leader sequences necessary for efficient expression and secretion of the light chain in mammalian cells. P530 provided the 1C2 V _{κ} II gene, and p347, which carries a human kappa light chain cDNA, was used as the source of the human kappa constant region gene.

The first stage of the process was to introduce a unique *Apa*L site into L15P61 at the junction between the leader sequence and the V_H gene of the CD3 heavy chain gene. This was achieved by site-directed mutagenesis, using oligonucleotides CM221 and CM222 to create a silent mutation (GTC → GTG) in the valine codon immediately upstream of the leader / V region junction (see figure 4 within Example 2). The mutagenesis reaction yielded a mutant of L15P61 (M611) that could be digested with *Apa*L I *Eco*R I to remove the V_H and C γ 1 regions of the CD3 heavy chain, whilst leaving the Kozak and leader sequences in place.

The second stage of the process was to isolate the 1C2 V _{κ} light gene from p530. This was done by using p530 DNA as the template for a PCR and using oligonucleotides CM223 and CM224 as the primers. The PCR generated a product of the expected size of 328 bp, which was subsequently purified by gel electrophoresis digested with *Apa*L and *Mae* II, then re-purified.

The next stage of the process was to isolate the human kappa constant region gene from p347. This was also achieved by PCR, using oligonucleotides CM225 and CM226 as the primers. The forward primer, CM226 was designed to introduce an *EcoR* I site at the 3' end of the C_k gene fragment. The reverse primer, CM225, was designed to hybridise across the V/JC junction of the p347 kappa light chain gene.

Not only did this oligo incorporate the *Mae* II site at the V/J junction, which was to be used in linking the 1C2 V_k gene to the human C_k gene, but it also introduced two amino acid changes into the p347 light chain framework region 4, to make it identical to the corresponding sequence in 1C2 V_k . Oligonucleotide CM225 was also used to delete the unwanted *Mae* II site that lies on the J/C junction of the p347 light chain gene. The PCR, performed as described in section 2.1.1.2.2 yielded a product of the expected size of 373 bp. After purification by gel electrophoresis, the product was digested with *Mae* II and *EcoR* I, and then re-purified.

In the final stage of the chimaeric light chain gene assembly process, *Apal* I – *EcoR* I-cut M611 vector DNA was ligated together with the *Apal* I – *Mae* II-cut 1C2 V_k II PCR product and the *Mae* II – *EcoR* I – cut C_k PCR product in a single ligation reaction. The ligation product was used to transform *E. coli* TG1, and RF DNA was prepared from 6 of the resulting plaques. Restriction enzyme digestion of the DNA using *Hind* III and *EcoR* I revealed that all six clones carried inserts of approximately 732 bp, the size expected for the assembled chimaeric gene. The inserts of two clones were sequenced using primers CM229 and CM230. Both contained a chimaeric 1C2 light chain gene, however, one clone was excluded from further study as it also contained a single nucleotide mutation in the constant region. The gene carried by M613 contained no unwanted mutations and so was used for further work.

4.2.1.3 Assembly of 1C2 chimaeric antibody expression vectors.

The system chosen for expressing the 1C2 chimaeric antibody genes was the Celltech GS system (Lonza Biologics). This system utilizes two plasmid expression vectors, pEE12 and pEE6.hCMV-*Bgl* II (pEE6). It is specially suited for the production of proteins such as antibodies that are composed of two heterogeneous polypeptides, and which must be expressed in mammalian cells. Both plasmids possess an immediate early gene enhancer and promoter from human cytomegalovirus, from which 'guest gene' (in this case, immunoglobulin gene) expression is driven. pEE12 also contains an expression cassette for glutamine synthetase (GS). This acts as a selectable marker for the plasmid when it is introduced into GS cells such as NS0 myeloma cells. GS cells that have incorporated the pEE12 plasmid are able to survive in glutamine-free medium that is supplemented with glutamate, whereas non-transferred cells die. The usual procedure for assembling a vector capable of directing the synthesis an antibody is to insert the heavy chain gene into pEE6 and to insert the light chain gene into pEE12. Each gene is therefore under the control of a separate copy of the hCMV promoter. The heavy chain expression cassette, including its hCMV promoter and poly A signal is then cut out of pEE6 and inserted into the pEE12-L chain vector, to create a single plasmid carrying both the heavy and light chain genes. This is the strategy that was used to create expression vectors for the production of the 1C2 γ 1 and α glycosyl γ 1 chimaeric antibodies.

In the first stage of expression vector construction, the genes for the 1C2 γ 1 and aglycosyl γ 1 chimaeric heavy chain were cut out of clone M609 and M610 respectively, using *Hind* III and *Eco*R I. The genes were separately ligated into *Hind* III-*Eco*R I-cut pEE6 vector DNA to produce pEE6 plasmid clones p531 and p532 (γ 1 and aglycosyl γ 1 respectively).

In parallel, the gene for the 1C2 chimaeric light chain was excised from M613 by *Hind* III - *Eco*R I digestion and ligated into *Hind* III - *Eco*R- cut pEE12 to produce clone p533. In the final stage of expression vector construction, the γ 1 and aglycosyl γ 1 heavy chain expression units were isolated by *Bgl* II - *Sal* I digestion from p531 and p532 respectively, and separately inserted into *Bam*H I - *Sal* I-cut p533. This created two plasmids, p534 and p535, capable of directing the expression of the 1C2 γ 1 and 1C2 aglycosyl γ 1 chimaeric antibodies respectively. When digested with *Hind* III and *Eco*R1, both of these plasmids were cut into 4 fragments as expected, with sizes of approximately 7.0, 2.4, 1.4 and 0.7 kbp. The 1.4 and 0.7 kbp fragments represent the heavy and light chain chimaeric genes. The heavy and light chains carried by the two plasmids were then sequenced using primers CM229 and CM230 for the light chain gene, and primers SN124, SN125, SN126, SN129, SN130 and CM231 for the heavy chain genes (Example 2, table 2.2). No sequence anomalies were detected (the sequences are shown in figures 3 and 4).

4.2.1.4 Transfection of NS0 cells with the 1C2 chimaeric antibody expression vectors.

The mouse myeloma cell line NS0 was chosen as the cell line for producing the 1C2 chimaeric antibodies. The cells are naturally GS, and so are suitable hosts for transfection with the Celltech GS expression system. Also their use for recombinant antibody production was already established in the laboratory, and antibody yields of up to 50 μgml^{-1} of immunoglobulin per ml of culture supernatant have been achieved in the past.

Two transfection experiments were carried out one with p534 DNA. The second with p535 DNA, the aim being to produce NS0 cell lines, which constitutively expressed either the 1C2, γ 1 or the 1C2 aglycosyl γ 1 chimaeric antibodies. In each transfection, approximately 1.5×10^7 NS0 cells were mixed with 50 μg of the appropriate plasmid DNA that has been linearized with *Sal* I. The cells were then subjected to electroporation and stable transfectants were selected for their ability to grow in glutamine-free selective medium in 96 well plates. Twelve days post transfection growth-positive wells were screened by ELISA for the presence of human IgG κ antibody in the culture medium. Of 35 growth-positive wells that arose from the p534 (1C2 γ 1 chimaeric antibody) transfection, 13 wells (37.1%) contained antibody at levels ranging from 4 to 32 μgml^{-1} . The remaining 22 wells were antibody positive, but contained less than 1 μgml^{-1} of antibody. Of 30 growth-positive wells that arose from the p535 (1C2 aglycosyl γ 1 chimaeric antibody) transfection, only 7 wells (23.3%) produced antibody at a concentration of between 1 and 2 μgml^{-1} . 21 of the remaining wells produced low level of antibody, and two wells were antibody negative. The antibody Campath 1 H (human IgG1 κ) was used as the reference antibody in the ELISA for IgG concentration estimation.

One well that contained only a single colony of cells, and which expressed the best possible levels of antibody, was selected from each transfection experiment. The wells chosen were 4C6 and 8A1 from the p534 ($\gamma 1$) and p535 (aglycosyl $\gamma 1$) transfections respectively. Whereas the amount of antibody detected in well 4C6 was around $16\text{--}32\ \mu\text{gml}^{-1}$ 8A1 only yielded $2\ \mu\text{gml}^{-1}$. The cells in each well were cultured in expanding volumes of medium over several weeks, until a total of 5 litres of 4C6 culture supernatant, and 12 litres of 8A1 supernatant had been collected. The supernatants from both cultures were tested for anti-RSV G protein binding activity using BSA-G as the capture antigen in ELISA. Both antibodies bound specifically to BSA-G, showing no reactivity against BSA control antigen.

They were also tested against, and shown to react specifically with RSV-infected HeLa cell antigen by ELISA. These results confirmed that the chimaeric 1C2 antibodies retained binding affinity for the RSV G protein.

Subsequently, the immunoglobulin in the 5 litres of 4C6 and 12 litres of 8A1 culture supernatant were separately purified on a Protein A Sepharose column. Approximately 113 mg of 4C6 and 39 mg of 8A1 antibody were recovered as estimated by protein assay (OD at 280 nm). The amount of 8A1 (aglycosyl $\gamma 1$) antibody recovered was nearly twice as high as expected, given the ELISA estimate of $2\ \mu\text{gml}^{-1}$ of human IgG in the 8A1 culture supernatant. The purified antibodies were dialysed against PBS pH 7.4, filter-sterilized then stored at -70°C at a protein concentration of $10\ \text{mgml}^{-1}$.

4.2.1.5 *SDS-PAGE and Western blot analysis of the 1C2 chimaeric antibodies*

To check the purity of the two chimaeric antibodies recovered after protein A-Sepharose column chromatography, $10\ \mu\text{g}$ of each antibody, along with $10\ \mu\text{g}$ of the original mouse 1C2 mAb, was run on a 5–18 % gradient SDS-polyacrylamide gel under reducing conditions. The gel was then stained with Coomassie blue. As expected two polypeptide bands of approximately 50 kD and 25kD were seen in the samples of mouse 1C2 mAb and chimaeric $\gamma 1$ mAb, corresponding to their heavy and light chain polypeptides. No other bands were detected in these two samples, indicating that they did not contain significant amounts of other protein contaminants.

4.3 Discussion

The nucleotide and amino acid sequences of the chimaeric antibodies are illustrated in Figure 7 (the heavy chain) and Figure 8 (the light chain).

4.3.1 *The glycosylated chimaeric antibody*

The 1C2 V_H and V_L genes were used to produce a mouse human chimaeric antibody consisting of the mouse 1C2 V_H and V_L regions linked respectively to human $\gamma 1$ heavy chain and human κ light chain constant regions.

The inventors found that such chimaeric mouse-human antibodies persist in the circulation approximately six times longer than murine IgG (which has a circulating half-life of only 15-30 hours). Accordingly these chimaeric mouse-human antibodies represent preferred antibodies according to the invention.

4.3.2 The aglycosyl chimaeric antibody

A second chimaeric antibody was produced, identical to the first apart from an Asn²⁹⁷ → Ala mutation in the $\gamma 1$ heavy chain C_H2 domain. As a result, this antibody lacked the heavy chain constant region N-linked carbohydrate attachment site and was therefore expressed in an aglycosyl form.

Both antibodies are preferred antibodies according to the invention.

EXAMPLE 5

In vivo immunoprophylaxis of RSV infection using the 1C2 γ 1 human chimaeric antibodies

This example was chosen as a means to illustrate the *in vivo* immunoprophylactic properties in RSV infection of the 1C2 γ 1 human chimaeric antibodies derived according to the present invention from the 1C2 hybridoma as described in section 1.1.1 and the two chimaeric antibodies identified in Example 3. Moreover, this example shows that the 1C2 γ 1 human chimaeric antibodies are suitable for the treatment of RSV infection *in vivo*.

5.1 Methods

5.1.1 *Intranasal infection of mice with RSV*

See section 1.1.2.1

5.1.2 *Intravenous injection of mice with monoclonal antibodies*

See section 1.1.2.2

5.1.3 *Lung homogenate preparation*

See section 1.1.2.3

5.1.4 *Virus titration (fluorescent focus assay)*

See sections 1.1.2.4 and 1.1.2.5

5.1.5 *Neutralisation test*

4-fold serial dilutions of the test antibody samples were prepared in maintenance medium in a sterile 96 well round bottomed plate at a final volume of 50 μ l per well. 25 μ l of RSV suspension (160 ffu per 25 μ l) was added to each well. When antibody neutralising activity was being tested in the presence of complement, 25 μ l of freshly reconstituted rabbit serum (Sigma, S7764) was also added to each well. This was replaced by 25 μ l of maintenance medium when antibody neutralising activity was being tested in the absence of complement. A 'no antibody and no complement' control, and a 'no antibody' control was included in each assay. The plate was incubated at 37°C for 1 hour in 5 % CO₂ in a moist box to allow virus neutralisation to take place. The quantity of infectious virus remaining in each well of antibody / virus (with or without complement) mixture was then assayed without further dilution on HeLa cell monolayers as described in section 1.1.2.4.

5.2 Results

To test the ability of the 1C2 chimaeric to prevent RSV infection *in vivo*, groups of 6 mice were injected intravenously with 1 mg of either mouse 1C2 mAb, γ 1 chimaeric mAb or aglycosyl γ 1 chimaeric mAb, then challenged 24 hours later by intranasal inoculation of 1.36×10^7 ffu of RSV. Control mice were given either PBS or the humanised YTH 12.5 mAb (specific for human CD3 antigen) as a human IgG1 isotype control, before challenge. When administering the chimaeric aglycosyl γ 1 mAb, each mouse in the group was injected with 1.9 mg of total protein from the protein A-Sepharose purified materials. This increased amount was to compensate for a quantity of contaminant protein that was present in the preparation. (A comparison by densitometry of the relative heavy chain polypeptide band intensities of the chimaeric γ 1 and aglycosyl γ 1 mAb indicated that the protein A purified aglycosyl γ 1 mAb actually contained 1.9-fold less chimaeric immunoglobulin than the purified γ 1 chimaeric mAb).

Four days after the intranasal challenge, the mice were killed and their lungs assayed for the presence of infectious RSV.

The results are shown in figure 5. All three groups of mice that had received either the original mouse or chimaeric 1C2 antibodies had significantly less RSV in their lungs than the mice given either PBS or the control CD3 mAb.

However, the protective effect produced by the original mouse 1C2 mAb and the chimaeric γ 1 mAb was significantly better than that produce by the chimaeric aglycosyl γ 1 mAb. No virus was recovered from the lungs of these first two groups of mice, whereas an average of 1.95×10^3 ffu/g of RSV was found in the lungs of mice treated with the aglycosyl γ 1 mAb. These results indicate that the protective activity of the chimaeric γ 1 mAb is comparable to that of the mouse 1C2 mAb. They also demonstrate that antibody Fc region function plays an important role in the protective efficacy of the 1C2 anti-G mAbs.

5.3 Discussion

These data illustrate that 1C2 mAb and humanised derivatives thereof are useful for treating RSV infections. In particular it demonstrates that activity of the antibody is improved by glycosylation. Accordingly, preferred antibodies for use according to the invention are glycosylated (e.g., chimaeric γ 1 mAb and glycosylated derivatives thereof).

The surprising nature of the invention is illustrated by the fact that *in vitro* viral neutralization assays (data not shown) suggested that 1C2 and humanized versions thereof had limited neutralizing properties. The efficacy of antibodies according to the invention was only fully realised when *in vivo* studies were performed.

These results also affirm the thoughts relating to the *in vivo* application of the scFv phage 1C2 antibody for the treatment of RSV infection as expressed in Example 3.

CLAIMS

1. An antibody, or a functional derivative thereof, against the G glycoprotein of Respiratory Syncytial Virus characterised in that a Variable Region comprises:

(i) a first domain containing at least one peptide sequence selected from:

- (a) Arg Ser Ser Gln Asn Ile Val His Scr Asp Gly Asn Thr Tyr Leu Glu (SEQ ID No. 1); or
- (b) Lys Val Ser Asn Arg Phe Ser (SEQ ID No. 2); or
- (c) Phe Gln Gly Ser His Ile Pro Trp Thr (SEQ ID No. 3)

derivable from an antibody V_L region; and

(ii) a second domain containing at least one peptide sequence selected from:

- (d) Asp Tyr Ala Met His (SEQ ID No. 4); or
- (e) Val Ile Ser Thr Tyr Tyr Gly Asn Pro Asn Tyr Asn Gln Lys Phe (SEQ ID No. 5); or
- (f) Ser Asp Met Ile Thr Ala Gly Gly Gly Tyr Ala Met Asp Tyr (SEQ ID No. 6)

derivable from an antibody V_H region.

2. The antibody, or a functional derivative thereof, according to claim 1 wherein the first domain comprises all three peptides of Seq. ID No. 1, 2 and 3; and the second domain comprises all three peptides of Seq. ID No. 4, 5 and 6.

3. The antibody, or a functional derivative thereof, according to claim 1 or 2 wherein the first domain comprises the V_L region of Figure 5 and the second domain comprises the V_H region of Figure 4.

4. The antibody, or a functional derivative thereof, according to any preceding claim comprising two identical light (L) chains and two identical heavy (H) chains wherein each chain comprises a Variable domain and at least one Constant domain and wherein the Variable domain of the light chain (V_L) comprises all three peptides of Seq. ID No. 1, 2 and 3 and the Variable domain of the heavy chain (V_H) comprises all three peptides of Seq. ID No. 4, 5 and 6.

5. The antibody, or a functional derivative thereof, according to claim 4 wherein the light chains and heavy chains are chimaeric proteins with Variable domains derived from non-human immunoglobulin and Constant domains from human immunoglobulin.
6. The antibody, or a functional derivative thereof, according to claim 5 wherein the Constant domain of each chain is from human IgG.
7. The antibody, or a functional derivative thereof, according to claim 5 or 6 wherein the Variable domain comprises the V_L region of Figure 5 and the V_H region of Figure 4.
8. The antibody, or a functional derivative thereof, according to claim 4 wherein the Variable domain comprises a human V_L region with all three peptides of Seq. ID No. 1, 2 and 3 grafted therein; and a human V_H region with all three peptides of Seq. ID No. 4, 5 and 6 grafted therein.
9. The antibody, or a functional derivative thereof, according to claim 8 wherein the Constant domains of each chain is from human IgG.
10. The antibody, or a functional derivative thereof, according to any one of claims 1 - 3 comprising an scFV fragment.
11. The antibody, or a functional derivative thereof, according to claim 10 with substantially the same amino acid sequence as depicted in Figure 3.
12. A DNA molecule coding an antibody Light Chain gene, or functional derivative thereof, characterised in that the DNA molecule comprises at least one nucleotide sequence selected from the group comprising:
 - (a) AGA TCT AGT CAG AAC ATT GTA CAT AGT GAT GGA AAC ACC
TAT TTA GAG (SEQ ID NO .7); or
 - (b) AAA GTT TCC AAC CGA TTT TCT (SEQ ID NO. 8); or
 - (c) TTT CAA GGT TCA CAT ATT CCG TGG ACG (SEQ ID NO .9).

13. The DNA molecule according to claim 12 comprising each of the nucleotide sequences of SEQ ID NO.s 7 – 9.
14. The DNA molecule according to claim 12 with the nucleotide sequence shown in Figure 5.
15. A DNA molecule coding an antibody Heavy Chain gene, or functional derivative thereof, characterised in that the DNA molecule comprises at least one nucleotide sequence selected from the group comprising:
 - (a) GAT TAT GCT ATG CAC (SEQ ID NO .10); or
 - (b) GTT ATT AGT ACT TAC TAT GGT AAT CCA AAT TAC AAC CAG AAG TTT (SEQ ID NO .11); or
 - (c) TCG GAT ATG ATT ACG GCC GGC GGC GGG TAT GCT ATG GAC TAC (SEQ ID NO .12).
16. The DNA molecule according to claim 15 comprising each of the nucleotide sequences of SEQ ID NO.s 10 – 12.
17. The DNA molecule according to claim 15 with the nucleotide sequence shown in Figure 4.
18. A DNA molecule comprising a DNA molecule according to the claim 12 linked, in-frame, to a DNA molecule according to claim 15.
19. An expression cassette or gene construct comprising a DNA molecule according to any one of claims 12 – 18.
20. An expression vector comprising a DNA molecule according to any one of claims 12 – 18.
21. A cell transformed with a DNA molecule according to any one of claims 12 – 18; an expression cassette according to claim 19; or an expression vector according to claim 20.

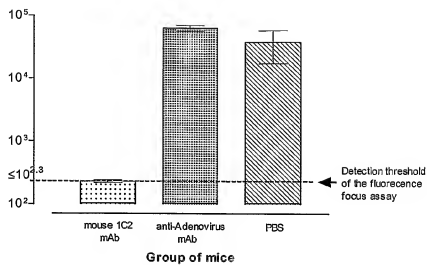
Fig.1

Fig. 2

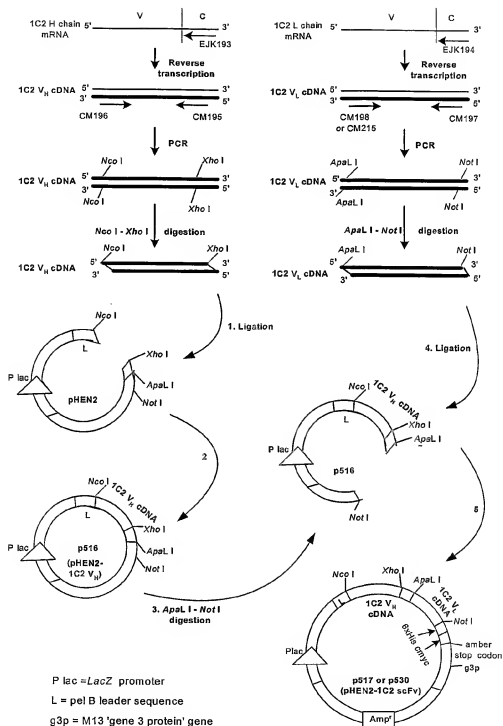


Fig. 3

```

      10      20      30      40
ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
|-----PelB leader sequence-----
MET Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu

      50      60      70      80      90
GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG CAG TCA GGG
|-----|-----PstI site
Ala Ala Gln Pro Ala MET Ala Gln Val Gln Leu Gln Gln Ser Gly

      100     110     120     130
CCT GAG GTG GTG AGG CCT GGG GTC TCA GCG AGG ATT TCC TGC AAG
Pro Glu Val Val Arg Pro Gly Val Ser Ala Arg Ile Ser Cys Lys

      140     150     160     170     180
GGA TCC GGC TAC ACA TTC ACT GAT TAT GCT ATG CAC TGG GTG AAG
Gly Ser Gly Tyr Thr Phe Thr Asp Tyr Ala MET His Trp Val Lys

      190     200     210     220
CAG AGT CAC GCA AAG AGT CTA GAG TGG ATT GGA GTT ATT AGT ACT
Gln Ser His Ala Lys Ser Leu Glu Trp Ile Gly Val Ile Ser Thr

      230     240     250     260     270
TAC TAT GGT AAT CCA AAT TAC AAC CAG AAG TTT AAG GGC AAG GCC
Tyr Tyr Gly Asn Pro Asn Tyr Asn Gln Lys Phe Lys Gly Lys Ala

      280     290     300     310
ACA ATG ACT GTA GAC AAA TCC TCC AGC ACA GCC TAT ATG GAA CTT
Thr MET Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr MET Glu Leu

      320     330     340     350     360
GCC AGA TTG ACA TAT GAG GAT TCT GCC ATC TAT TAC TGT GCA AGA
Ala Arg Leu Thr Tyr Glu Asp Ser Ala Ile Tyr Tyr Cys Ala Arg

```

Fig. 3 cont.

```

      370      380      390      400
TCG GAT ATG | ATT ACG GCC GGC GGC GGG TAT | GCT ATG GAC | TAC TGG
Ser Asp MET Ile Thr Ala Gly Gly Gly Tyr Ala MET Asp Tyr Trp

      410      420      430      440      450
GGC CAA GGG ACC ACG | GTC ACC GTC | XhoI site | GGA GGC GGT TCA
      TCG AGT GGT
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Ser
      -----linker-----

      460      470      480      490
GGC GGA GGT GGC TCT GGC GGT AGT CCA CAG | GAT GTT TTG | ATG ACC
      -----
Gly Gly Gly Gly Ser Gly Gly Ser Ala Glu Asp Val Leu MET Thr

      500      510      520      530      540
CAG ACT CCT CTC TCC | CTG CCT GTC | AGT CTT GGA GAT CAA GCC TCC
Gln Thr Thr Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser

      550      560      570      580
ATC TCT TGC AGA TCT AGT | CAG AAC ATT GTA CAT AGT GAT | GGA AAC
Thr Ser Thr Arg Arg Pro Gln Asp Ile Val His Ser Thr Gly Asp

      590      600      610      620      630
ACC TAT TTA GAG TGG | TAC CTG CAG AAA CCA GGC CAG TCT CCA AAG
Thr Tyr Leu Glu Trp Tyr Leu Gln Leu Pro Gly Gln Ser Pro Lys

      640      650      660      670
CTC CTG ATC | TAC AAA GTT TCC AAC CGA TTT | TCT GGG GTC | CCA GAC
Leu Leu Ile Tyr Lys Val Ser Asp Arg Phe Ser Gly Val Pro Asp

      680      690      700      710      720
AGG TTC AGT GGC AGT | GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile

```

Fig. 3 cont.

```

      730           740           750           760
AGC AGA GTG | GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC TTT CAA
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln
      770           780           790           800           810
GGT TCA CAT ATT CCG TGG ACG TTC GGT GGA GGG ACC AAG CTG GAG
Gly Ser His Ile Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu

      820           830           840           850
ATC TCC GCG GCC GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA GAA
NotI site |----6xHis-tag-----|
Phe Ser Ala Ala Ala His His His His His His Gly Ala Ala Glu

      860           870           880           890
CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG
-----cmvc-tag-----|
Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala stop

```

Fig. 4

CAG GTG CAG CTG CAG CAG TCA GGG
Gln Val Gln Leu Gln Gln Ser Gly

CCT GAG GTG GTG AGG CCT GGG GTC TCA GCG AGG ATT TCC TGC AAG
Pro Glu Val Val Arg Pro Gly Val Ser Ala Arg Ile Ser Cys Lys

GGA TCC GGC TAC ACA TTC ACT GAT TAT GCT ATG CAC TGG GTG AAG
Gly Ser Gly Tyr Thr Phe Thr Asp Tyr Ala MET His Trp Val Lys

CAG AGT CAC GCA AAG AGT CTA GAG TGG ATT GGA GTT ATT AGT ACT
Gln Ser His Ala Lys Ser Leu Glu Trp Ile Gly Val Ile Ser Thr

TAC TAT GGT AAT CCA AAT TAC AAC CAG AAG TTT AAG GGC AAG GCC
Tyr Tyr Gly Asn Pro Asn Tyr Asn Gln Lys Phe Lys Gly Lys Ala

ACA ATG ACT GTA GAC AAA TCC TCC AGC ACA GCC TAT ATG GAA CTT
Thr MET Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr MET Glu Leu

GCC AGA TTG ACA TAT GAG GAT TCT GCC ATC TAT TAC TGT GCA AGA
Ala Arg Leu Thr Tyr Glu Asp Ser Ala Ile Tyr Tyr Cys Ala Arg

TGG GAT ATG ATT ACG GCC GGC GGC GGG TAT GCT ATG GAC TAC TGG
Ser Asp MET Ile Thr Ala Gly Gly Gly Tyr Ala MET Asp Tyr Trp

GGC CAA GGG ACC ACG GTC ACC GTC
Gly Gln Gly Thr Thr Val Thr Val

Fig. 5

CAG GAT GTT TTG ATG ACC

Gln Asp Val Leu MET Thr

CAG ACT CCT CTC TCC CTG CCT GTC AGT CTT GGA GAT CAA GCC TCC

Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser

ATC TCT TGC AGA TCT AGT CAG AAC ATT GTA CAT AGT GAT GGA AAC

Ile Ser Cys Arg Ser Ser Gln Asn Ile Val His Ser Asp Gly Asn

ACC TAT TTA GAG TGG TAC CTG CAG AAA CCA GGC CAG TCT CCA AAG

Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys

CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA GAC

Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Asp

AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile

AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC TTT CAA

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln

GGT TCA CAT ATT CCG TGG ACG TTC GGT GGA GGG ACC AAG CTG GAG

Gly Ser His Ile Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu

ATC TCC

Ile Ser

Fig. 6

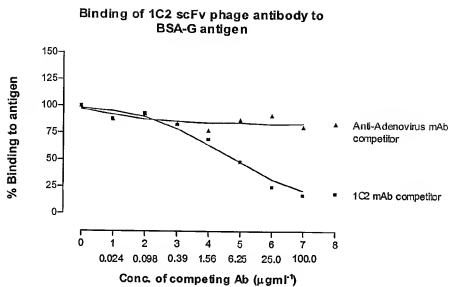


Fig. 7

```

      10           20           30           40
      |           |           |           |
AAG CTT GCC GCC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG
EcoRI site Kozak seq |-----Ig leader peptide sequence-----
                      MET Gly Trp Ser Cys Ile Ile Leu Phe Leu

      50           60           70           80           90
      |           |           |           |           |
GTA GCA ACA GCT ACA GGT GTC CAC TCC CAG GTC CAA CTG CAG CAG
-----|-----PstI site
Val Ala Thr Ala Thr Gly Val His Ser Gln Val Gln Leu Gln Gln

      100          110          120          130
      |           |           |           |
TCA GGG CCT GAG GTG GTG AGG CCT GGG GTC TCA GCG AGG ATT TCC
Ser Gly Pro Glu Val Val Arg Pro Gly Val Ser Ala Arg Ile Ser

      140          150          160          170          180
      |           |           |           |           |
TGC AAG GGA TCC GGC TAC ACA TTC ACT GAT TAT GCT ATG CAC TGG
Cys Lys Gly Ser Gly Tyr Thr Phe Thr Asp Tyr Ala MET His Trp

      190          200          210          220
      |           |           |           |
GTG AAG CAG AGT CAC GCA AAG AGT CTA GAG TGG ATT GGA GTT ATT
Val Lys Gln Ser His Ala Lys Ser Leu Glu Trp Ile Gly Val Ile

      230          240          250          260          270
      |           |           |           |           |
AGT ACT TAC TAT GGT AAT CCA AAT TAC AAC CAG AAG TTT AAG GGC
Ser Thr Tyr Tyr Gly Asn Pro Asn Tyr Asn Gln Lys Phe Lys Gly

      280          290          300          310
      |           |           |           |
AAG GCC ACA ATG ACT GTA GAC AAA TCC TCC AGC ACA GCC TAT ATG
Lys Ala Thr MET Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr MET

      320          330          340          350          360
      |           |           |           |           |
GAA CTT GCC AGA TTG ACA TAT GAG GAT TCT GCC ATC TAT TAC TGT
Glu Leu Ala Arg Leu Thr Tyr Glu Asp Ser Ala Ile Tyr Tyr Cys

```

Fig. 7 cont.

370 380 390 400

GCA AGA TCG GAT ATG ATT ACG GCC GGC GGC GGG TAT GCT ATG GAC

Ala Arg Ser Asp MET Ile Thr Ala Gly Gly Gly Tyr Ala MET Asp

410 420 430 440 450

TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GCC TCC ACC

BstBII site

Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr

460 470 480 490

AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC AAG AGC ACC

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr

500 510 520 530 540

TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC

Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe

550 560 570 580

CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC AGC

Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser

590 600 610 620 630

GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC TAC

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr

640 650 660 670

TCC CTC AGC AGC (G) GTC GTG ACC GTG CCC TCC AGC AGC TTG GGC ACC

BstBII site deleted

Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr

680 690 700 710 720

CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG

Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys

Fig. 7 cont.

```

      730          740          750          760
      |          |          |          |
GTG GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr

      770          780          790          800          810
      |          |          |          |          |
TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val

      820          830          840          850
      |          |          |          |
TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu MET Ile Ser Arg

      860          870          880          890          900
      |          |          |          |          |
ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp

      910          920          930          940
      |          |          |          |
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT
Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His

      950          960          970          980          990
      |          |          |          |          |
AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
      SacII site
Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
      (Ala)

      1000          1010          1020          1030
      |          |          |          |
CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn

      1040          1050          1060          1070          1080
      |          |          |          |          |
GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala

```

Fig. 7 cont.

1090 1100 1110 1120
 CCC ATC GAG | AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
 Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu

 1130 1140 1150 1160 1170
 CCA CAG GTG TAC ACC | CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG
 Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
 1180 1190 1200 1210
 AAC CAG GTC | AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC
 Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser

 1220 1230 1240 1250 1260
 GAC ATC GCC GTG GAG | TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC
 Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn

 1270 1280 1290 1300
 TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC
 Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe

 1310 1320 1330 1340 1350
 CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG
 Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly

 1360 1370 1380 1390
 AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC
 Asn Val Phe Ser Cys Ser Val MET His Glu Ala Leu His Asn His

 1400 1410 1420 1430
 TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA ATT C
 Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys stop
 EcoRI site

Fig. 8

```

      10          20          30          40
      AAG CTT GCC GCC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG
EcoRI site Kozak seq -----Ig leader peptide sequence-----
                        MET Gly Trp Ser Cys Ile Ile Leu Phe Leu

      50          60          70          80          90
      GTA GCA ACA GCT ACA GGT GTG CAC TCC GAT GTT TTG ATG ACC CAG
-----ApaLI site-----
      Val Ala Thr Ala Thr Gly Val His Ser Asp Val Leu MET Thr Gln

      100         110         120         130
      ACT CCT CTC TCC CTG CCT GTC AGT CTT GGA GAT CAA GCC TCC ATC
      Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser Ile

      140         150         160         170         180
      TCT TGC AGA TCT AGT CAG AAC ATT GTA CAT AGT GAT GGA AAC ACC
      Ser Cys Arg Ser Ser Gln Asn Ile Val His Ser Asp Gly Asn Thr

      190         200         210         220
      TAT TTA GAG TGG TAC CTG CAG AAA CCA GGC CAG TCT CCA AAG CTC
      Tyr Leu Glu Trp Tyr Leu Glu Leu Pro Glu Gln Ser Pro Arg Leu

      230         240         250         260         270
      CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA GAC AGG
      Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Asp Arg

      280         290         300         310
      TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC
      Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser

      320         330         340         350         360
      AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC TTT CAA GGT
      Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly

```

Fig. 8 cont.

```

      370      380      390      400
TCA CAT ATT | CCG TGG MaeII ACG TTC GGC GGC GGC ACC AAG CTG GAA ATC
Ser His Ile Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile

      410      420      430      440      450
AAG CGT ACT GTG GCT GCA CCA TCT | GTC TTC ATC TTC CCG CCA TCT
Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser

      460      470      480      490
GAT GAG CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC CTG CTG
Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu

      500      510      520      530      540
AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG AAG GTG GAT
Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp

      550      560      570      580
AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC ACA GAG CAG
Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln

      590      600      610      620      630
GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG ACG CTG
Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu

      640      650      660      670
AGC AAA GCA | GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC GAA GTC
Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val

      680      690      700      710      720
ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC AGG
Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg

```

Fig. 8 cont.

730
|
GGA GAG TGT TAG GAA TTC
EcoRI site
Gly Glu Cys stop

Fig. 9

